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**Quantitative and qualitative analysis of pyrogenic carbon by a revised benzene polycarboxylic acid method, applying high performance liquid chromatography for separation**

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## Sammanfattning

Pyrogen kol (eng. pyrogenic carbon) är en produkt av ofullständig förbränning av fossila bränslen och biomassa, det är allmänt förekommande i alla miljömatriker på jorden. Den globala värmebalansen, jordens albedo, klimatförändringar, och kolets kretslopp är några av forskningsområden där pyrogen kol spelar en nyckelroll inom olika processer och mekanismer. Pyrogen kol kan användas för att datera historiska skogsbränder, och tros förbättra jordmånernas kvalitet. Ett flertal olika analysmetoder används för närvarande, men resultaten varierar avsevärt både mellan olika metoder och mellan olika laboratorier som tillämpar samma metod. En ny metod för kvantifiering och kvalificering av halten pyrogen kol i en uppsättning av olika standardiserade referensmaterial, samt i svartjorden (chernozem), har tillämpats och utvärderats i denna studie. Metoden är baserad på mätning av molekylära markörer – aromatiska karboxylsyror, och inkluderar oxidation av proverna med salpetersyra, filtrering, frystorkning, och separation med högupplösande vätskekromatografi (HPLC). Ovanligt låga halter av pyrogen kol uppmättes i träkol, upp till 8 storleksordningar lägre jämfört med andra studier; låga halter uppmättes även i svartjorden, upp till 52 gånger lägre jämfört med andra kontrollstudier som använde sig av samma metod. Låga koncentrationer tros bero på ofullständig oxidation och plötslig upplösning av aromatiska karboxylsyror under förbehandlingen av proverna. Underskattningen av halten pyrogen kol som berodde på felaktig kalibrering har inträffat för proverna hexansot och svartjord, oavsett så är innehållet av pyrogen kol i hexansot någorlunda i linje med andra studier. Pyrogen kol har upptäckts i melanoidin, materialet som vanligen används för negativ kontroll och som normalt ej skall innehålla spår av pyrogen kol. Det pyrogena kolets karaktär har bedömts genom att mäta fördelningen av de enskilda aromatiska karboxylsyror. Högst andel, 78 %, av fullständigt substituerad aromatisk carboxylsyra, har uppmätts i hexansot.

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## Part 1.

# Quantitative and qualitative analysis of pyrogenic carbon by a revised benzenepolycarboxylic acid method

## Abstract

Pyrogenic carbon or black carbon is a product of incomplete combustion of fossil fuels and biomass; it is ubiquitous in all environmental matrices on Earth. Global radiation budget, albedo, climate changes, global carbon budget are a few areas where pyrogenic carbon plays a key role for different processes and mechanisms. Pyrogenic carbon can be used to date historical events of forest fires, and is reported to significantly improve the quality of soils. A number of different analysis methods are currently in use, but quantification results suffer of large discrepancies both between different methods, and between laboratories applying the same method. A new molecular marker, benzene polycarboxylic acid (BPCA) method for quantification and qualification of pyrogenic carbon content in a set of pyrogenic standard reference materials, and in chernozem soil, has been studied and evaluated in this assignment. BPCA method includes oxidation of the samples with HNO<sub>3</sub>, filtration, freeze-drying, and separation by high performance liquid chromatography (HPLC), equipped with diode array detector (DAD). Very low pyrogenic carbon content was measured in chars, up to 8 orders of magnitude lower compared to other studies; and in chernozem samples, up to 52 times lower compared to control studies applying the same method. Low concentration of pyrogenic carbon is believed to be a result of incomplete oxidation and unexpected destruction of BPCA molecules during sample preparation. Underestimation of pyrogenic carbon content due to calibration range has been encountered for hexane soot and chernozem soil samples, however measured pyrogenic carbon content in hexane soot is somewhat in line with other studies. Pyrogenic carbon quality was assessed by contribution of individual BPCA:s. Pyrogenic carbon was detected in melanoidin, a negative control material, which should not contain any fire derived carbon. Highest proportion of fully substituted B<sub>6</sub>CA (78%) was found in soot, the most condensed material.

## Keywords

Pyrogenic carbon, black carbon, elemental carbon, benzene polycarboxylic acids molecular marker, geochemistry, high performance liquid chromatography, incomplete combustion particles, soil science, carbonaceous refractory material, forest fires,

## 1. Aim

The main objective with this study was to perform quantitative and qualitative analysis of a set of environmental samples, applying a new BPCA molecular marker method, Part 1 of this study. Materials included in the study are chernozem soil sample; positive control, pyrogenic carbon rich materials such as grass char, wood char, and high condensed hexane soot; and negative control melanoidin. The results have been compared to other studies. Another aim for this work was to set up a lab, maintain and prepare the HPLC instrumentation required for the pyrogenic carbon analysis, which is briefly described in Part 2.

## 2. Introduction

Since the Rosen et. al. (1978), reported in their study that *graphitic refractory particles* found in the urban dust strongly absorbs light the interest to investigate further those particles has grown since then. Pyrogenic carbon is globally abundant throughout all the environments on Earth, it is present in the soils, igneous, metamorphic and sedimentary rocks, ocean water column, on land, in the soils, lacustrine and marine deposits. Pyrogenic carbon appears in the atmosphere as the aerosol (Goldberg, 1985). Pyrogenic carbon has unique properties such as high heat resistance, slow turn over time, and ability to absorb light. Due to a long residence time pyrogenic carbon has a significant impact on the global carbon budget (Masiello, 2004). Pyrogenic carbon is the most effective light absorbing compound found in the atmosphere, and therefore it is of great interest to investigate its impact on atmospheric radiative budget (Bond, 2013). Anthropogenic nanoparticles of historic importance may contain traces of pyrogenic carbon (Wiedemeier, 2013; Ziolkowski, 2009).

### 2.1.1 Pyrogenic carbon budget

Black carbon originates from a broad range of different combustion processes and most part of the sources are anthropogenic. Anthropogenic release of black carbon into the environment is mostly a result of incomplete combustion of fossil fuels or forest fires (Goldberg, 1985). Forest fires are the largest contributors of pyrogenic carbon to the atmosphere worldwide. Annual global emission of black carbon is estimated at 7500 Gg yr<sup>-1</sup>, but the uncertainty is very large, between 2000 and 29000 Gg yr<sup>-1</sup> (Bond, 2013). The residence time of the pyrogenic carbon in soils and sediments is reported to be between 2400 and 13900 years (Kuhlbusch, 1996). The estimation of the global carbon budget expects a negative result abbreviated as missing carbon sink, that is about more than 1 Gt C year<sup>-1</sup>, the number varies between different studies (Bond 2013). Schmidt et. al. (2005), reports, that up to 20% of that missing carbon sink could be counted as charcoal pyrogenic carbon. Pyrogenic carbon takes part of many biogeochemical processes on Earth (Poot, 2009).

### 2.1.2. Role of pyrogenic carbon in the environment

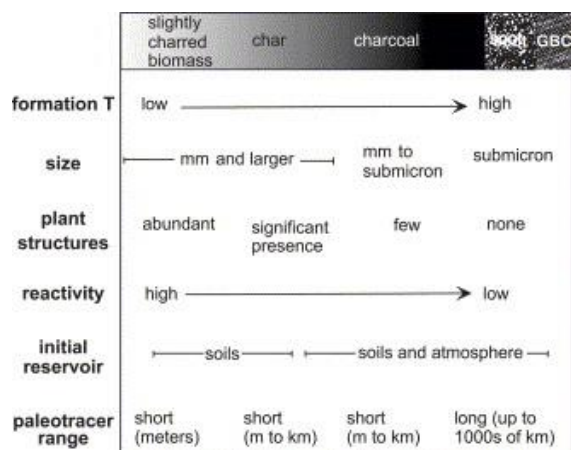
Goldberg (1985) suggests that black carbons possibly influence weather and climate on Earth due to its unique properties. Pyrogenic carbon particles present in the atmosphere as aerosols absorb significant amount of solar radiation and transform energy into heat. Unique properties of pyrogenic carbon make it important for investigation of its role in the atmospheric radiative budget, since the concentration of such a strong light absorber in the atmosphere is relatively high. The presence of the pyrogenic carbon in the atmosphere influences also cloud formation. Presence of black carbon on snow and ice causes positive climate forcing, since the black carbon effectively absorbs solar radiation, it may alter the albedo, and increase the melting of snow and ice (Bond, 2013; Jacobson, 2000; Hansen, 2000). Pyrogenic carbon is probably a second largest contributor to the global warming after carbon dioxide, due to its absorbing properties and interaction with the sunlight (Jacobson, 2000; Hansen, 2000; Shrestha, 2010). Since it has an impact to the climate forcing it is important to investigate the exact role of black carbon in the Earth's climate processes. Previous quantitative studies of black carbon had led to biases and great uncertainty in the results it is therefore of great importance to improve the analysis methods (Bond, 2013). In the climate science, carbon dioxide budget is dependent on the reliable quantification of carbon pools and sinks in the environment. While the CO<sub>2</sub> emissions are well studied, the pyrogenic carbon, which is a significant pollutant in the atmosphere, has received less

attention and possess a high global warming potential (Bond, 2005). On the other hand, presence of the PyC in soil, due to its recalcitrance and long residence time it may play a significant role as the sequester of carbon (Shackley, 2009). Charcoal as a sink in the global carbon budget is mentioned by Goldberg (1985), where the size of the reservoirs and fluxes must be estimated in order to evaluate the contribution of charcoal to the global budget of carbon. Mass balance estimation is also dependent on the degradation rates of refractory carbon material, which influences the flux of pyrogenic carbon. It is suggested that pyrogenic carbon persists in the environment for a very long time, resulting in major impact on the Earth's slow-carbon pools (Masiello, 2004). About 80% of all pyrogenic carbon produced ends up in soils with a long residence time up to hundreds and thousands of years. Which means pyrogenic carbon is an important global carbon sink (Forbes, 2006; Hammes, 2007). Different carbonaceous materials, including pyrogenic carbon in soils are reported to interact with persistent organic pollutants (POP) by sorption mechanisms, thus playing an important role for the accumulating, transport and risk management for those hazardous materials (Koehlmans, 2006). Schmidt et. al. (1999), describes how the presence of charred organic matter (COC) affect the physical and chemical properties of the soils. Schmidt et. al. (1999), concluded that physical, chemical and biological properties of soil, and pedogenesis (soil development) are affected by the presence of COC. Refractory properties of BC make it possible to be preserved in the soil/sediment for a very long time. Comparing the transportation rates with the rate of production published by different authors, Forbes et. al. (2010), suggests that significant amounts of black carbon are exposed to decomposition. The mechanisms and rates of pyrogenic carbon degradation are not well understood yet and need be studied further to understand the role of pyrogenic carbon in the global carbon budget. Other importance of black carbon is as the trace of ancient forest fires, estimate ancient climate on the Earth, and to help to create a complete picture of the climate on the Earth during previously years (Schmidt, 2001). Presence of black carbon in the sediment can be a signature of higher oxygen content in the ancient Earth atmosphere (Schmidt, 2000).

### 2.2.1 Definition of pyrogenic carbon

The general definition of pyrogenic or black carbon is not precise due to the lack of defined structure. Characterization of pyrogenic carbon is complicated because the material is a product of combustion; it has different sources, such as pyrolyzed plants, wood and fossil fuel; and its coexistence with other types of carbon such as organic carbon derived from plants that has not been exposed for combustion and inorganic carbon. To elucidate the property differences and complexity of the pyrogenic carbon material a combustion continuum model has been introduced by Hedges et. al. (2000). The model represents the changing of pyrogenic material properties with rising combustion temperature and is shown in the Figure 1. Schmidt et. al. (2000), suggests that it is more convenient to describe specific chemical and physical properties that are typical for pyrogenic carbon. Black carbon is a common term, mainly used in sediment and atmospheric science; it describes carbon rich material, derived from charred plants residues, or significantly graphitized soot. However this term has limitations, since the term describes only visual properties, and it is imprecise since it covers rather a part of, or a complete range of different materials derived from the combustion continuum (Hammes, 2007). Bond et. al. (2013) recommend to use term refractory black carbon, since the term black carbon has not been used regularly in quantification studies. It is preferable to use term pyrogenic carbon, PyC is used, since it exactly refers to the origin of the carbon, or which processes the material has been undergone,

and refers more to chemical and physical properties, rather than only physical property as term black carbon does (Weidemeier, 2013).



**Figure 1.** Combustion continuum model for pyrogenic carbon substance. Adapted from: Masiello (2004).

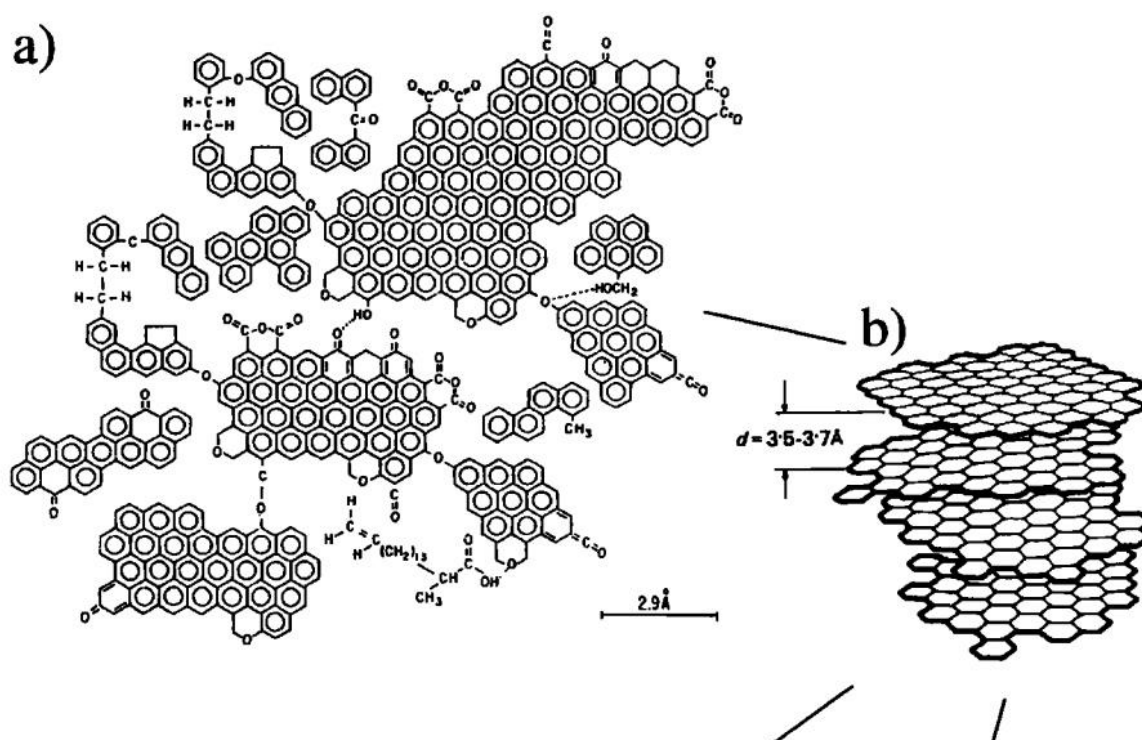
### 2.2.2. Chemical and physical properties of pyrogenic carbon

Pyrogenic carbon is thermally and chemically relatively stable, resistant to biological degradation; it lacks a defined chemical structure (Masiello, 2004; Brodowski, 2005a). Particles of pyrogenic carbon are small and very black (Jacobson 2000; Hansen et. Al. 2000). A common feature for PyC is the aromatic structure of the material, although the chemical and physical properties vary significantly between charcoals and soots, depending on the precursor of the combusted material, the extent of the combustion continuum, combustion temperature, the absence or presence of the oxygen, and weathering condition after the formation of particles (Goldberg, 1985; Masiello, 2004). Pyrogenic carbon particles are present in a broad range of size and shape, ranging from nm spherule soot objects and up to mm particles of char with retained structure of precursor biomass (Fernandes, 2003). Black carbon particles contains significant amount of carbon, about >60%, and are rich in sulfate ( $\text{SO}_4^{2-}$ ), other significant elements are hydrogen (H), oxygen (O) and nitrogen (N) (Goldberg, 1985; Bond, 2013). The carbon concentration increases during the condensation, increased charring and soot production (Hammes, 2007). The presence of the aromatic rings, and relatively few function groups in the molecular structure of PyC makes it resistant to degradation (Dai, 2005). Black carbon is refractory, which means that the structure of pyrogenic carbon is stable up to very high temperatures, up to 4000 K (Goldberg, 1985). Pyrogenic carbon is insoluble in water and organic solvents, such as methanol or acetone. Black carbon has a strong ability to absorb visible light (Bond, 2013). Pyrogenic carbon matter has very high surface to volume ratio which provides it strong affinity to other non-polar compounds, pollutants in soil, such as other aromatic substances (Mattila, 2008).

### 2.2.3 Formation of pyrogenic carbon

Black carbon, or pyrogenic carbon is formed during the incomplete combustion of carbon rich fuels, in reduced or anoxic environments, during exothermic reactions at temperatures between 280° and 500°C, in oxygen deficiency, with increased aromaticity along with higher charring temperatures (Schmidt, 2001; Masiello, 2004; Bond, 2013). Cellulose molecules in the organic matter are gradually transformed into aromatic molecules during the combustion (Masiello, 2004). Complex chemical reactions lead to formation of polycarboxylic aromatic

hydrocarbon (PAH) molecules which are the precursor of the black carbon. Fast coagulation of PAH-molecules results in accumulation of matter into spherules-like structures of 10 nm in diameter with very high C:H ratio, structure is shown on Figure 2. Those spherules stick to each other into chain-like aggregates. Spherule structure differ black carbon from planar structure of graphite. The spherule structure of black carbon possesses a very irregular morphology which equips pyrogenic carbon matter with active sites that provide adsorption of other chemical substances (Zhang, 2008). After the emission to the atmosphere, other gaseous and organic compounds rapidly condense on the surface of spherule aggregates. Schmidt et. al. (2000), describes two different ways of BC formation, distinguishing between volatiles and solid residuum, forming soot and char respectively. Soot is a product from hot gases present in the flames. Char formation is referred to combustion partly in flame and partly during slow, low-temperature, flameless combustion. More char material is produced at lower combustion temperatures, below 300°C, and production of volatiles increases at the combustion temperatures above 300°C (Schmidt, 2000). Soot is much less reactive and has no remnants of plant structure which is significant for chars. The rests of the biomass in chars makes it possible to identify the precursor fuel type (Shrestha, 2010). Chars and charcoal are able to decompose at higher rates than soot under the oxidation, microbial destruction, and photo-oxidation. Elmquist et. al. (2006), describes that soot is more refractory than char due to its greater condensation rate at higher temperatures, lower internal micro-porosity, and lower O/C ratio. The decay processes are complex and decomposition rates are different for various types of charcoal (Schmidt, 2001). Both soot and chars usually co-exist during the combustion and thus both are present at the same time in the sediment (Goldberg, 1985).



**Figure 2.** Pyrogenic Carbon chemical structure (a) and basic structural units (b). Adapted from Schmidt et. al. 2000

### 2.3.1. Analysis difficulties

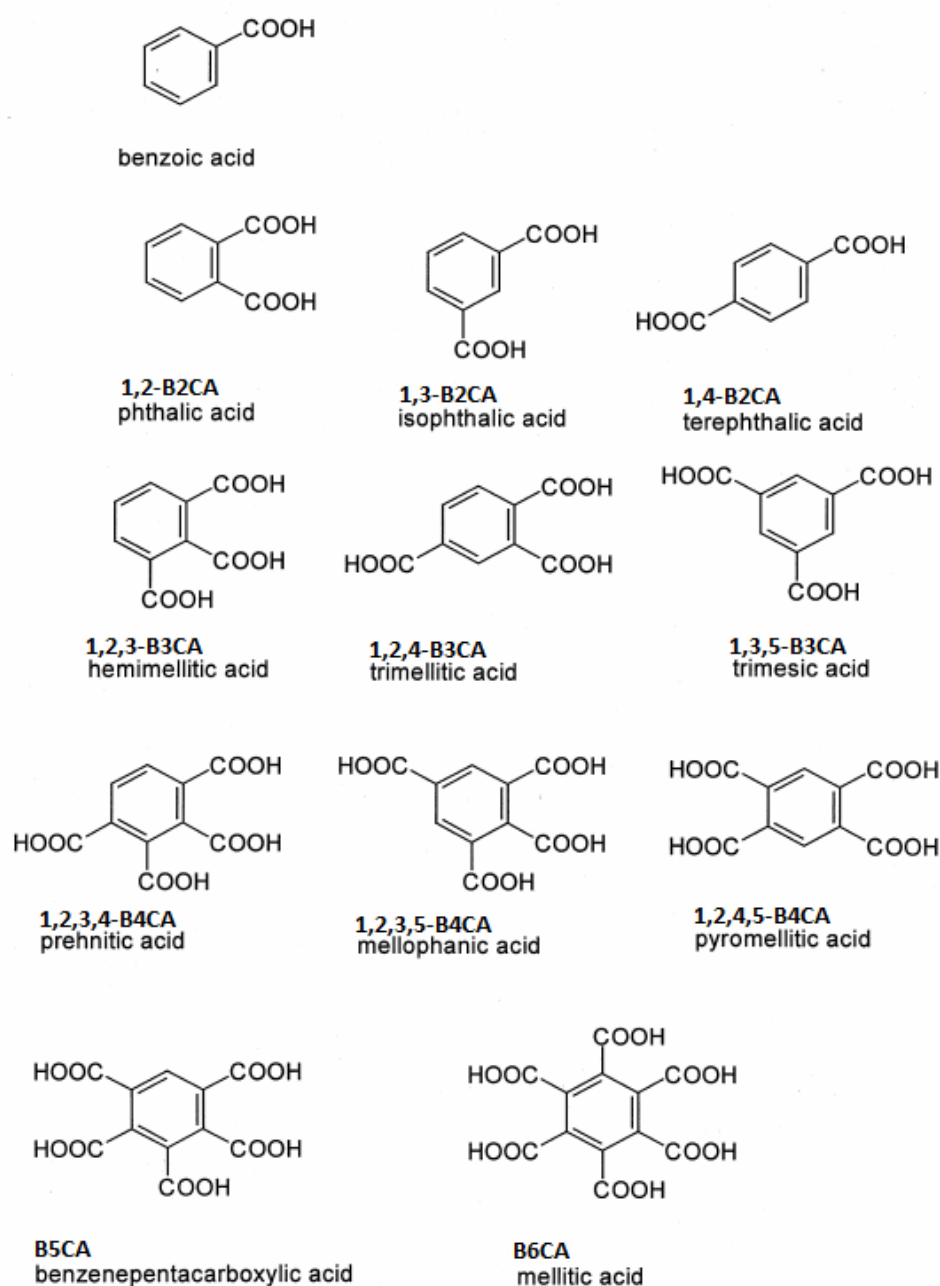
The analysis methods of the pyrogenic carbon has been under development for recent decades, especially since the first deeper review of black carbon by Goldberg was published in 1985. At first the methods to quantify pyrogenic materials was developed and adapted to investigate atmospheric samples. Eventually it has been evaluated that quantification of pyrogenic carbon in sediments is not suitable with the methods customized for atmospheric research, leading to large amount of discrepancy of the results (Hammes, 2007). Methods suitable for analysis of PyC in sediment have been under development since then. Since the pyrogenic carbon possess a variable chemical and physical properties, its quantification in different environmental matrices has led to modeling of quantification methods adjusted to those differences. Those methods have been adjusted with respect to the purpose of the studies, such as different environmental purposes, or analysis of pyrogenic carbon derived from different combustion continuums (Hammes, 2007). The broad variety of different quantification methods has inevitably led to a large discrepancy of the results. One of among other reasons to this variation is that different methods measure different parts of the combustion continuum, since the broad variety of combustion bi-products formation as a result of different precursor fuels, temperature and combustion conditions (Masiello, 2004; Hammes, 2007; Poot, 2009). Quantitative analysis of black carbon is also complicated due to the presence of other interfering compounds in the material or the impurity of PyC (Bond, 2013). The absence of consensus for quantification makes it difficult to incorporate pyrogenic carbon into global carbon budget estimation (Forbes, 2006). The development of methods for quantification of pyrogenic carbon mainly considers a fact that the pyrogenic material is highly resistant to the degradation and oxidation. In order to quantify the pyrogenic carbon the main step for the analysis is to separate carbon of the pyrogenic origin from the organic carbon and inorganic carbon that are present in the sample. To accomplish this elimination, different methods apply diverse techniques. The most convenient methods that are in use are thermal-chemical (oxidation and heating); chemical (extraction); optical methods; spectroscopic (nuclear magnetic resonance, NMR) and molecular marker methods (Hammes, 2007). Schmidt et. al. 2001, concludes that those different analytical methods to quantify pyrogenic carbon have a large deviation in the results, up to a factor of 500 for the same sample analyzed. This huge discrepancy of the results is explained as an impact of definition difficulties of pyrogenic carbon, and tendency to rather overestimate or underestimate the pyrogenic carbon depending on the intrinsic properties for each method. A very common chemical-thermal method in use to extract pyrogenic carbon soot from soils and sediments, has been reported to form pyrogenic carbon artefacts in PyC-free soils, and thereby an overestimation of the PyC concentration (Simpson, 2004), and also there may be a risk of losing pyrogenic carbon particles during the pretreatment, due to many handling steps (Nguyen, 2004). Chemical methods apply oxidation to differentiate pyrogenic carbon matter at different stability (charring) stages. Chemical methods tend to overestimate pyrogenic carbon, some variations of the method are reported to be very laborious, and also loss of pyrogenic carbon occurs during the many handling stages that are required (Poot, 2009). NMR-technique has a tendency to underestimate the pyrogenic carbon due to specific properties of the signal reading in the instrumentation (Masiello 2004). Shrestha, 2010, states that in the atmospheric studies, most common methods that are in use to study PyC are based on the thermo-optical analysis, which suffers from both underestimation and overestimation. Hammes et. al. (2007), reports that some of chemical properties, such as specific surface area

and oxidative kinetics, can sometimes overlap between soot and char which makes them difficult to distinguish in different forms of pyrogenic carbon, which leads to methodological issues prior the assessment of PyC. To solve those methodological problems a BPCA molecular marker method is suitable since it enables the analysis of the products that are derived from a broad range of combustion continuum, since the content of BPCA:s is directly linked to the pyrogenic content in the material. Contribution of different BPCA molecules indicates different chemical and physical properties of the precursor fuel and combustion conditions (Wiedemeier, 2013). However, according to Hammes (2007), the molecular marker method applying BPCA might underestimate the pyrogenic carbon content in highly condensed aromatic structures such as soot, which was also detected by Schneider char pyrolysis study. In the large ring trial, Hammes (2007), also concludes that BPCA method suffer of the artificial BPCA formation in the negative control materials such as shales and coals, that doesn't contain any pyrogenic material. Also the reproducibility in the ring trial has gained attention, since the results varied by factors of two between different laboratories using the same BPCA method. Those biases are thought to be partly addressed to the many handling steps during the sample preparation. To achieve less biased results, the HPLC separation has an advantage compared to GC, since there is no need for the derivatization of the sample prior the separation, and thus less handling steps. Concentration of the pyrogenic carbon in char increases with the rising charring temperature, however, Schneider, et. al. (2010), describes a decline of the black carbon concentration in the samples that were heated at higher temperatures 800-1000 °C. This sudden decrease is an indication of the incomplete digestion with nitric acid, thus means that highly condensed structures will lead to an underestimation of the pyrogenic carbon in samples.

### 2.3.2. BPCA-method background

The original BPCA-method was firstly presented by Glaser (1998), and applied gas chromatography for the separation. The BPCA molecular marker method makes it possible for both quantitative and qualitative analysis of pyrogenic carbon (Glaser, 1998; Brodowski, 2005b, Schneider 2010). This original method by Glaser (1998) included a pretreatment step with hot hydrochloric acid (HCl) prior the digestion with hot nitric acid (HNO<sub>3</sub>) in order to convert PyC to individual BPCA molecules, Figure 3; percolation through resin to remove polyvalent ions; and derivatization prior the separation on a gas chromatography (GC) equipped with flame ionization detector (FID). Initially a citric acid has been introduced by Glaser (1998), to be used as an internal standard for the BPCA method. However, Schneider et. al. (2010), described that the citric acid is deteriorated in strong acid conditions, and suggested to be replaced by a more BPCA similar phthalic acid, that is more stable during the acid digestion. The pretreatment step was later reported to result in artefact formation of BPCA and overestimation of pyrogenic carbon in samples (Brodowski 2005b). In the same article, Brodowski, et. al. (2005b), recommends to replace HCl with trifluoroacetic (TFA) acid. In order to quantify pyrogenic carbon in seawater, the BPCA-method was adjusted to perform separation by high performance liquid chromatography (HPLC) combined with diode array detector (DAD) instead of GC (Dittmar, 2008). This new method didn't include any filtering steps and could eliminate derivatization procedure required by GC separation. HPLC method also allowed a reduced amount of sample that was needed for the analysis, up to 10 times less, compared to GC (Dittmar, 2008). Initially the separation for the HPLC method was performed at a higher pH 8. Schneider et. al. 2011, compared GC and HPLC and adjusted the HPLC method to achieve faster separation, but the separation of some BPCA:s was not sufficient

enough. Both Dittmar et. al. (2008) and Schneider et. al. (2011) did not use any filtration procedures after the digestion, the samples were only dried under N<sub>2</sub> and residue was dissolved in methanol/water solution prior the HPLC separation.



**Figure 3.** Chemical structures of benzene polycarboxylic acid (BPCA)s and their abbreviations. Adapted from: Glaser et. al. (1998), p. 813, with added abbreviations used in this text.

However, elimination of the filtration steps is only appropriate for analysis of pure organic samples such as soot and char reference materials. The analysis of the soil matter requires elimination of different organic and inorganic substances (Wiedemier 2013). The oxidation of pyrogenic carbon into BPCA:s is not complete during the digestion. Originally a conversion factor of 2,27 was introduced by Glaser et. al. (1998). Later, Brodowski et. al. (2005b) reported that the conversion factor for charcoals should be 4,5, but this conversion factor

would lead to a large overestimation if applied to chernozem soil samples. Consequently Schneider et. al. 2010, strongly advises to report the results without any use of the correctional factor, since the same correction factor cannot be used for different types of samples. Overall HPLC-separation is proven to generate more reliable results with less discrepancy. Wiedemeier et. al. 2013 have presented an improved BPCA-method to quantitatively and qualitatively analyze BC. Wiedemeier et. al. (2013) compared GC to HPLC separation, and confirmed previous conclusions that GC underestimates PyC content due to incomplete derivatization, and that GC produces higher deviation of the results gained from multiple samples. This new and revised method is used in this study for further evaluation and analysis of pyrogenic carbon content in environmental samples.

### 3. Materials and method

#### 3.1. Materials for the analysis

To improve the method development and the comparability of different methods, Black Carbon Steering Committee (BCSC) has developed a set of different pyrogenic carbon reference materials, it is strongly advised to use those in the pyrogenic carbon quantification studies (Schmidt, 2003; Hammes, 2007). Standardized use of the pyrogenic reference materials is of vital importance because carbon content may vary significantly between different environmental samples. In this study, three different groups of materials has been used, 1) four different standard reference materials that are pyrogenic carbon rich and produced in laboratory, 2) one interference material that doesn't contain any products of combustion as false-negative marker, and 3) one pyrogenic carbon containing environmental matrices or chernozem soil; summary is shown in Table 1.

**Table 1.** Pyrogenic carbon reference materials used in this study. Organic carbon content is a mean value for results from different laboratories, brackets value represents standard deviation. Adapted from: Hammes et. al. (2007). Chars, soots and melanoidin: Black carbon standard material. Supplier: Geography Dept., Biogeochemistry Group, University of Zürich, Switzerland

Material	Production Method	Organic Carbon, g kg <sup>-1</sup> mass	Motivation
Melanoidin	Product of the reaction between urea and glucose heated at 90°C for 30 days	543,3 (30,1)	Negative control, doesn't contain pyrogenic carbon. Potentially interfering material. Negative control.
Char 1. Lignocellulosic char from risotto rice	Grass ( <i>Oryza sativa</i> ) char, produced at 450°C for 5 hours, in nitrogen atmosphere	591,4 (32,0)	Black carbon standard reference material. Pyrogenic carbon derived from grass biomass. Positive control.
Char 2. Lignocellulosic char from chestnut wood	Chestnut ( <i>Castanea sativa</i> ) produced at 450°C for 5 hours, in nitrogen atmosphere	744,4 (45,6)	Black carbon standard material. Pyrogenic carbon derived from wood biomass. Positive control
n-Hexane soot	Produced and collected in an open n-hexane flame	921,3 (32,9)	Soot in atmospheric aerosols, (EC), Positive control.

Chernozem	Light sandy soil collected at Hildesheim, Germany, contains 19% clay	19,3 (0,9)	Pyrogenic carbon in soil matrix, low clay content
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Chernozem samples were prepared at Stockholm University, and two control samples prepared at Zürich by Wiedemeier et. al. (2013) was used for comparison. All of the samples, except for the two Chernozemic soils samples from Switzerland which were already prepared and ready for HPLC-analysis, were treated exactly the same way by BPCA-method according to Wiedemeier et. al. (2013). Soot, chars and melanoidin black carbon reference material samples were analyzed with 3 replicates, and chernozemic soil were analyzed with 5 replicates.

### 3.1.2 Char

Charred biomass, in this case with origins from risotto rice (*Oryza sativa*) and chestnut wood (*Castanea sativa*) to investigate different origin materials will produce different levels of BPCA in the analysis. Chars are characterized by its appearance of carbon-rich aromatic residues, has a size variation of 1 to 100  $\mu\text{m}$ . (Hammes, 2007). Charcoals used in this study were charred at 450 °C. Somewhat higher concentration of the PyC is usually detected in wood char compared to grass char. Higher lignin content in wood is a possible explanation why larger amounts of PyC is detected in wood char (Schenider, 2011).

### 3.1.3. Soot

Soot produced in the gas phase by incomplete combustion of hexane, is used as soot standard material. Soot is identified as carbon-rich condensates (Hammes, 2007). The lower O/C and H/C ratios are significant for the higher degree of condensation of the soot. Usually charred material and soot particles are both found at the same time in the environmental samples (Schmidt 2001). Both soot and chars can exhibit the same properties, depending on the formation states, which makes them difficult to distinguish from each other and brings further difficulties for detection of pyrogenic carbon (Hammes, 2007).

### 3.1.4. Melanoidin

Melanoidin is a dark brown substance consisting of an amorphous and insoluble polymers produced by Millard reaction with presence of amino acids and carbonates. Benzig-Purdie and Ripmeester, 1983, suggest that melanoidin have similar chemical properties as natural organic matter. Melanoidin is used as a negative control because it doesn't contain any pyrogenic material (Hammes 2007). The chemistry of the melanoidin is proposed to be of the same character as for the natural organic matter. However, not all the organic matter, that is not of pyrogenic origin, in soils and sediments, has a similar chemical properties with melanoidin, which makes melanoidin less effective as a negative control (Hammes 2007). Brodowski et. al. (2005b), suggests that all aromatic structures in soils that origins from melanoidin, should be counted as pyrogenic carbon, since the polyaromatic C-structures are a result of reactions that occur at high temperatures and is therefore a result of fire or burning, and Maillard reaction (formation of melanoidin) occurs seldom in the absence of fires. Hammes et. al. (2007), reports that melanoidin yielded large detection of black carbon using optical methods, which is due to a dark color of melanoidin. Hammes et. al. (2007), concluded that melanoidin may contain a small amount of black carbon markers, such as condensed aromatic structures,

with expected concentrations 30-50 times lower than PyC content found in chars, using BPCA-method. Brodowski (2005b), applied Glasers (1998), original BPCA method that includes acid (HCl or TFA) treatment of samples with HCl prior digestion with HNO<sub>3</sub>, and GC for quantification, could measure the BPCA to a mean yield of 13 mg BPCA per g melanoidin. Presence of BPCA could be due to the pretreatment with HCl or TFA, it has been previously reported that pre-treatment of this kind may induce the artificial formation of BPCA (Brodowski, 2005b). However, Glaser (1998), reports that no BPCA was recovered from melanoidin material.

### 3.1.5. Chernozemic soil

Chernozemic soil sample was sent to Stockholm University from Zürich. For this study the amount of soil weighed for the BPCA-analysis was calculated with the respect to the given amount of org Carbon (org. C) or total organic carbon (TOC) content according to the data reported by Schmidt et. al. (1999). The chernozem soil sample was originally sampled at the depth region 20-60 cm in Hildesheim-Braunschweig region in the middle northern part of Germany known for high abundance of chernozem soils. The organic C content is 17,8 g kg<sup>-1</sup>, and soil has rich silt content, 74,6 mass-%; and relatively high clay content, 23,9 mass-%; minor part of sand, 1,6 mass-%. The base saturation or cation exchange capacity was reported to be relatively high, and soils showed presence of bioturbation. According to the results from NMR presence of large amounts of un-substituted carbon was linked to the high amount charred organic carbon (COC), which is resistant to photo-oxidation (Schmidt, 1999). Schmidt et., al. (1999) have found significant concentration of COC in Chernozemic soil using a photo-oxidation method combined with confirmation through the SEM. COC content was reported up to 45 % of the total amount of organic matter (TOC), which is equivalent to 8 g kg<sup>-1</sup> of soil content.

### 3.2. Method description

Complex polymer structure of PyC is broken up by digestion with 65% nitric acid (Sigma Aldrich) into smaller parts of BPCA-monomers (Figure 3). Under digestion with nitric acid, pyrogenic particles are oxidized and hydrophilic carboxyl groups (-COOH) are formed on surface of molecules. The BPCA-monomers are later filtrated to remove carbonates, kerogen, humic substances, and other organic material (Schmidt, 2000), and analyzed by high performance liquid chromatography (HPLC). General information for the BPCA-method by Wiedemeier et. al. 2013 is presented in Table 1.

**Table 2.** General specifics for the benzene polycarboxylic acids method applied to quantify and characterize pyrogenic carbon

Reference	Oxidation			Detection		
	Time	Temperature	Acid	Instrument	Column	Mobile Phase
Wiedemeier (2013)	8 Hours	170°C	65% HNO <sub>3</sub>	HPLC-DAD, Waters 2695/Waters 996,	Agilent Proshell 120 SB-C18, 100 mm x 4,6 mm.	A, Acetonitrile B, Phosphoric acid, buffered with NaH <sub>2</sub> PO <sub>4</sub>

Previously prepared HPLC separation module Waters 2695 was used to perform separation of BPCA:s. The instrument was equipped with Waters 996 diode array detector (DAD) for the peak identification, and a column heater. Column that was applied was of the same model as used by Wiedemeier et. al. (2013), an Agilent Proshell 120 SB-C18, length is 100 mm, and inner diameter 4,6 mm. Absorbance spectra range was set at 190-400 nm. Wavelength of 240 nm was used to obtain chromatograms for quantification and peak analysis. Peak area quantification was performed in the Mass Lynx 4,1 software. Calculation of the concentration, statistical analyses (average, calibration curves, coefficient of variation of the results), and diagrams were completed on Microsoft Excel 2010. Different injection volumes of 5  $\mu$ l, 10  $\mu$ l, 15  $\mu$ l, 20  $\mu$ l and 25  $\mu$ l, was tested on the chernozem soil samples to see the response on HPLC. The rest of the samples and all the BPCA-standards were injected with a volume of 10  $\mu$ l.

### 3.2.1. Sample Preparation - Digestion

All of the samples, except for the two Chernozemic soils samples from Switzerland which were already prepared and ready for HPLC-analysis, were treated exactly the same way by BPCA-method according to Wiedemeier. Et. al. 2013. Soot, chars and melanoidin black carbon reference material samples were analyzed with 3 replicates, and chernozemic soil were analyzed with 5 replicates. Soil samples and pyrogenic reference materials were weighed and marked in the glass tubes, prior the nitric acid digestion. Approximately 0,001 g of each reference materials (soot, chars, melanoidin), and 0,05 g of chernozem soil were weighed on a AG245 Melter Toledo (max 41g/210g, d=0,0mg/0,1mg) and placed into microwave glass tubes, and 2 ml of concentrated, 65% nitric acid (Sigma-Aldrich) was added. Glass tubes with sample solutions were left for digestion in a Milestone UltraWave Single Reaction Chamber Microwave Digestion System for 8 h at 170°C. Inside the instrument, vials were placed in to the PTFE-vessel, and the carousel was lowered in to a chamber of stainless steel filled with 100 ml of ultrapure water. When the chamber was sealed for operation under high pressure, a proper run program was chosen on the operation panel. Instrument was programed with respect to NO<sub>3</sub>, at 170°C, according to vapor versus temperature diagram to sustain a stable operation. Pressure during the digestion was set at 80bar. It is important to use caps with aperture on the top to avoid pressure build up inside the sample vials. According to a stability test described by Schneider et. al. 2010, the BPCA samples, after HNO<sub>3</sub> digestion, can be stored up to one month. After the digestion, samples with dissolved BPCA:s were filtered with 5 ml ultrapure water through the cellulose filter Whatman CAT No. 1820-030, GF/A Size 3,0 cm. The total volume of the solution after first filtration was about 7 ml.

### 3.2.2. Sample Preparation – Removal of the apolar compounds

To prevent HPLC-column from contamination, the apolar compounds must be removed from the sample solutions. A resin-column was prepared with a glass reaction tube with Teflon frit at the bottom and filled with 2-3 cm resin, Dowex 50W x8, diethyl-benzene polymer with ethenylbenzene, sulfonated Dowex R. Resin-columns were protonated with 6 ml diluted nitric acid, and cleaned with approximately 3-4 ml ( $V_{\text{resin}} \times 4$  ml) of ultrapure water (Milli-Q, Millipore Corporation) to pH 5. The pH was systematically checked with litmus paper. Before the dilution, molarity of nitric acid was calculated by the density, 1,39 kg l<sup>-1</sup> (given on flask). Molar mass of the nitric acid is 63,01 g mol<sup>-1</sup>. HNO<sub>3</sub> concentration is 65%, the molar concentration was calculated by

$$0,65 \times 1390g \frac{903,5 gHNO_3}{63,01g mol^{-1}} = 14,3 mol l^{-1} \quad (\text{Eq 1})$$

Molarity of the nitric acid was calculated to 14,3 M and therefore the acid was diluted 20 times (1ml HNO<sub>3</sub> with 19 ml ultrapure water) to protonate the resin. Diluted nitric acid molarity was 0,168 M. After digestion and first filtration the residues with BPCA:s were poured in to the resin columns and rinsed with 12 ml of ultrapure water in to larger glass vials of 35 ml volume. The final volume of BPCA sample solutions was then about 19 ml. Afterwards glass reaction tubes with cellulose filter (Whatman CAT No. 1820-030, GF/A Size 3,0 cm) was prepared for further filtration and the solution was stored in 50 ml glass vials with caps. Resin columns were cleaned and protonated before the filtration of the next sample because it gets polluted by cations. Percolation was performed over a vacuum box, vacuum valve-connections were rinsed with a mix of methanol (CH<sub>3</sub>OH), dichloromethane (DCM, Cl<sub>2</sub>CH<sub>2</sub>) and hexane (C<sub>6</sub>H<sub>14</sub>). It is important to maintain slow dripping during filtration to avoid dissolution or contamination with resin compounds.

### 3.2.3. Sample preparation - freeze-drying

After the filtration over resin-column samples were freeze dried. Sample solutions were poured into a freeze-drying flasks and they were placed into a rotating drum in the instrument for freeze-drying, Scanvac CoolSafe™. The temperature for the freeze-drier must be chosen below materials triple-point to ensure that rather sublimation than melting will occur. Due to the leaking and malfunctioning of the freeze-drier, drying of sample solutions took too long time, about 10-12 hours, sometimes longer. An attempt to freeze samples with liquid nitrogen was tried out. Inside the fume hood, liquid nitrogen was poured into a beaker, later glass tubes with sample solution was lowered into the nitrogen beaker for about 0,5 min until the sample was solid and frozen, and stored in a freezer. Special gloves were used to handle liquid nitrogen for safety reason.

### 3.2.4. Sample preparation - C18-filtration

After freeze-drying, the residue in the bottom of glass vials was re-dissolved in a small amount of methanol/water (1:1) solution. As little as possible, or about 1 ml of methanol solution was used for washing, until the residue was completely dissolved. Vials were rinsed with methanol/water 3 times. Subsequently the re-dissolved residue was pipetted into prepared DSC-18 cation exchange cartridges over a vacuum stand, and washed with 2-3 ml ultrapure water into reaction vials for further elimination of apolar compounds. DSC-18 needs to be conditioned with a solution that is the same or similar to the sample matrix (according to manual). The DSC-18 tube was filled with ultra-pure water with about half of the tube volume of 6ml, water was then filtrated, and later tubes were filled with methanol/water solution. A little of solvent was remained on the top of the sorbent surface to sustain a good contact between sample solution and the hydrophobic solid phase of the sorbent. Once used DSC-18 tubes were discarded to avoid contamination. After C18 filtration, the sample solutions in reaction tubes were fixed on a heating plate with silica sand stand, and dried with nitrogen gas (N<sub>2</sub>) until complete dryness. Last step was to re-dissolve the dried residue in 1 ml ultrapure water and place the solution in to HPLC-vials, at this point the samples were ready for the chromatography analysis.

### 3.3. Calibration with BPCA standards

For the calibration curve a set of BPCA-standard solutions were prepared. For this study following BPCA-derivates were used for standards:

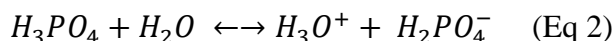
1. Hemimellitic acid, 1,2,3-BPCA (98%) – standard 1
2. Trimellitic acid, 1,2,4-BPCA (99%) – standard 2
3. Trimesic acid, 1,3,5-BPCA (95%) – standard 3
4. Pyromellitic acid, 1,2,4,5-BPCA (96%) – standard 4
5. Benzepentacarboxylic acid – standard 5
6. Mellitic acid (99%) – standard 6

Approximately 5 g of each internal standard were weighed into glass tubes and dried over night over night in an oven at 105°C. After drying, 500 mg of standard 1, 2, and 3, and 100 mg of standards 5, and 6, were weighed and poured in to volumetric flasks, 50 ml for Standards 1, 2, 3, and 4, and 25 ml for standards 5 and 6. Flasks with standards were filled up with ultrapure water. Standards 1, 2, 3, and 4 did not dissolve completely. The solubility was lowest for the standard 1 and was increasing by number carboxylic groups attached to the benzene ring. To increase dissolution, flasks with the standard solutions were sonicated for 60 min. After the sonication, all standards except standard 3 dissolved completely. Standard 3, 1,3,5-benzenetetracarboxylic acid, showed precipitation after one day and was further diluted 5 times to a 250 ml volumetric flask. Standard 3 was sometimes pre-heated up to 48°C to dissolve precipitated BPCA-salt. After about a week in storage, standard 4 started to precipitate salt at the bottom of the volumetric flask; solution was heated and sonicated before the preparation of the BPCA standard mixtures. Initially BPCA-standards were analyzed at concentrations 20, 60, 100, 140 and 180 µg ml<sup>-1</sup> per injection. Later the standards were run at concentrations of 20, 80, 160, and 200 ng per injection. It was then decided to run a mixture of standards at concentrations of 25, 50, 75, 100 and 200 ng to create a 5 point calibration curve with a range. BPCA standard mixtures were injected together with every batch prior the samples, totally 3 (except individual BPCA injections). Unfortunately the standard mixtures were not prepared correctly, so the concentration of individual BPCA:s per injection was 2, 4, 6, and 16 ng. Prehnitic and Mellophanic (two different B4CA:s) acids were not available. Since the amount of B2CA:s is expected to be very low in all types of materials, under the detection limit, they are not included in the study. Furthermore, Ziolkowski et. al. 2009, advises to not include B2CA:s in the analysis to avoid the measurement of BPCA:s that are derived from material that is not of pyrogenic origin, such as lignin.

### 3.4. Mobile phases

#### 3.4.1. Preparation of the mobile phases

To prepare Mobile phase A, 2ml of 85% phosphoric acid (Sigma-Aldrich) dissolved was dissolved in 1000 mL ultra-pure water and buffered with NaH<sub>2</sub>PO<sub>4</sub> to pH 1,2, under constant stirring on a magnet plate, and vacuum filtered over the 0,2 µm thin and 47 mm diameter membrane filter to remove possible impurities, as described by Wiedemeier (2013). The amount of NaH<sub>2</sub>PO<sub>4</sub> was calculated according to Henderson Hasselbalch-equation (Eby, 2003),



The pKa1 value is derived from the reaction formula above to

$$pH = pKa + \log \frac{[H_3O^+] * [H_2PO_4^-]}{[H_3PO_4]} \quad (\text{Eq 3})$$

The variables and the equation were written in excel (Appendix) and the value of  $k_{a1}$  was calculated with different settings of parameters. The calculated amount of  $\text{NaH}_2\text{PO}_4$  needed to buffer the solution to pH 1,2 was 0,2 g. The actual amount of  $\text{NaH}_2\text{PO}_4$  exceeded the theoretical (calculated) amount by 0,6-1,6 g. Directly after pouring of 10 ml phosphoric acid into 400 ml ultrapure water mixture had pH 1,077-1,089. For mobile phase B pure acetonitrile (Sigma-Aldrich) was used.

### 3.4.2. Calibration of pH-meter

The pH-measurement for the preparation of the mobile phase 1 was carried out on Lab 850 Schott Instruments. Calibration of the pH-meter needs to be performed in the interval of the expected pH that is going to be measured, in this case pH 1,2. Calibration solution for pH 1 was ordered, before that the calibration was performed in three steps for pH points 10, 7 and 4. Unreliable condition of the instrument made it necessary for an external pH control, which was performed with litmus paper, Duotest pH 1,0-4,3 Macherey-Nagel. Litmus test showed that the pH was in the range of 1,0-1,6, closest to pH 1,3. It is important to have the same temperature of the measured medium as the temperature during the calibration of the electrode since the  $pK_a$  is affected by temperature. To protect the electrolyte it must be washed with ultrapure water before and after each the pH-measurement, and immediately placed in the solution of 3M potassium chloride (KCl) solution to hydrate and prevent leaking.

### 3.4.3. Mixing gradient for mobile phase

The mobile phase gradient for the HPLC separation was the same as by Wiedemeier et. al. 2013, with minor adjustments of time during the mixing, and total runtime is 5 min longer to assure complete flushing of the sample rests through the column. Mobile phase gradient is presented in the Table 3, below.

**Table 3.** Mobile phase gradients, mobile phase A is pure acetonitrile; mobile phase B is orthophosphoric acid buffered with  $\text{NaH}_2\text{PO}_4$  to a pH 1,2

Time (min)	Mobile phase A (%)	Mobile Phase B (%)
0	0,5	99,5
5	0,5	99,5
25	30	70
26	95	5
28	95	5
29	0,5	99,5
35	0,5	99,5

## 3.5. Calculations

### 3.5.1. Amount of BPCA:s

The amount of the BPCA:s in each sample was calculated using the straight line function of calibration curves based on the peak areas for injections of BPCA standards at different concentrations. The total amount of BPCA:s were calculated as a sum of all the BPCA molecule markers (B6CA, B5CA, B4CA:s, and B3CA:s). The BPCA:s total amount was then used to calculate amount of pyrogenic carbon in each sample.

### 3.5.2. Qualitative analysis

The relative contribution of BPCA:s in the sample is related to the chemical properties of the pyrogenic carbon and the grade of the charring. If the sample yields a high distribution of fully substituted B6CA it indicates a highly condensed structure of the precursor material, since the B6CA formation occurs from the aromatic rings that are completely surrounded by other aromatic rings. Less substituted BPCA:s are produced when the aromatic ring is surrounded by only two other aromatic rings. The larger distribution of less situated BPCA:s indicates that the precursor material is more oxidized and not fully condensed as when the amount of B6CA is dominating (Ziokowski, 2009). The relative contribution of the BPCA molecules in a sample indicates the original size of the aromatic spherules of the pyrogenic origin (Schneider, 2010).

### 3.5.3. Calculation of Pyrogenic Carbon

**Table 4.** Chemical properties of different benzene polycarboxylic acids used for the calculation of pyrogenic carbon in samples

Abbreviation	Molecular Weight, g mol <sup>-1</sup>	C weight, g mol <sup>-1</sup>	O weight, g mol <sup>-1</sup>	H weight, g mol <sup>-1</sup>	C-ratio
Mellitic Acid	342,168	144,132	191,988	6,048	0,421
1,2,3,4,5-Benzenpentacarboxylic Acid	298,159	132,121	159,99	6,048	0,443
1,2,4,5-Pyromellitic Acid	254,15	120,11	127,992	6,048	0,473
1,2,4-Trimellitic Acid	210,141	108,099	95,994	6,048	0,514
1,2,3-Hemimellitic Acid	210,141	108,099	95,994	6,048	0,514
1,3,5-Trimesic Acid	210,141	108,099	95,994	6,048	0,514

Previously calculated carbon ratio (Table 4) was used to calculate carbon content in each BPCA for every sample. The sum of the BPCA carbon content was then used to calculate total amount of BPCA in the vial by

$$\frac{m_{BPCA} (g)}{V_{injected} (L)} * V_{HPLC\ vial} (L) \quad (Eq\ 4)$$

Where  $m_{BPCA}$  is amount of all BPCA detected per sample in g,  $V_{injected}$  is injection volume in L, and  $V_{HPLC\ vial}$  is volume of the BPCA extraction solution in the HPLC vials. Values for total BPCA:s carbon content were calculated in  $\mu\text{g}$  per vial. Later the weighed sample amount, 50 mg for soil and 1 mg for Pyrogenic carbon reference materials, and data for the organic carbon content in different materials in g per kg derived from Hammes et. al. 2007, was used to calculate the amount of carbon content per weighed sample [mg], as

$$m_{sample} (g) * C_{org\ C} (g\ kg^{-1}) \quad (Eq\ 5)$$

Where  $m_{sample}$  is sample mass in g, and  $C_{org\ C}$  is concentration of organic carbon in g per kg. Finally the amount of BPCA-carbon per sample [ $\text{g}\ \text{kg}^{-1}$ ] was calculated.

$$\frac{m_{BPCA\ vial}}{m_{C\ sample}} \quad (Eq\ 6)$$

Where  $m_{BPCA\ vial}$  is the amount of BPCA in HPLC vial in  $\mu\text{g}$ , and  $m_{C\ sample}$  is the organic carbon content per weighed sample in mg, which is equal to g BPCA-carbon per kg sample.

The missing B4CA standards, 1,2,3,4-prehnitic acid and 1,2,3,5-mellophanic acid, were compensated by times two of the detected 1,2,4,5-pyromellitic acid. For the compensation it

was assumed that the amount of individual B4CA:s is about the same for all three of them, the assumption is based on the results from Wiedemeier et. al. 2013, Fig. 1, p. 248, where the peaks for three individual B4CA:s have similar peak height and size. The compensation is not optimal, but eliminates the range of underestimation of pyrogenic carbon content in the samples.

### 3.5.4. Limit of detection

Limit of detection describes the lowest amount of analyte that is significantly different from the blank. It can be also calculated by multiplication of the mean value of a blank signal times three (Harris, 2003).. The but since the signal of BPCA:s in the blanks was significantly lower than the signal for BPCA standards injected at the lowest concentration, 2 ng, this point was considered as detection limit.

## 4. Results

The first analysis of individual BPCA:s standards gave the results suffering from peak broadening, and it later showed that the spectrum of the peaks didn't even matched with the spectrums from later runs. Some times when HPLC stopped running no data on injected standards could be acquired. Perhaps impurities in the injector or else in the system resulted in distorted peaks, broad peaks with large tailing. After the clogging issues of the system was resolved the shape of the peaks was significantly improved, peak broadening ceased. The data for all HPLC analysis is summarized in the Table 5 below.

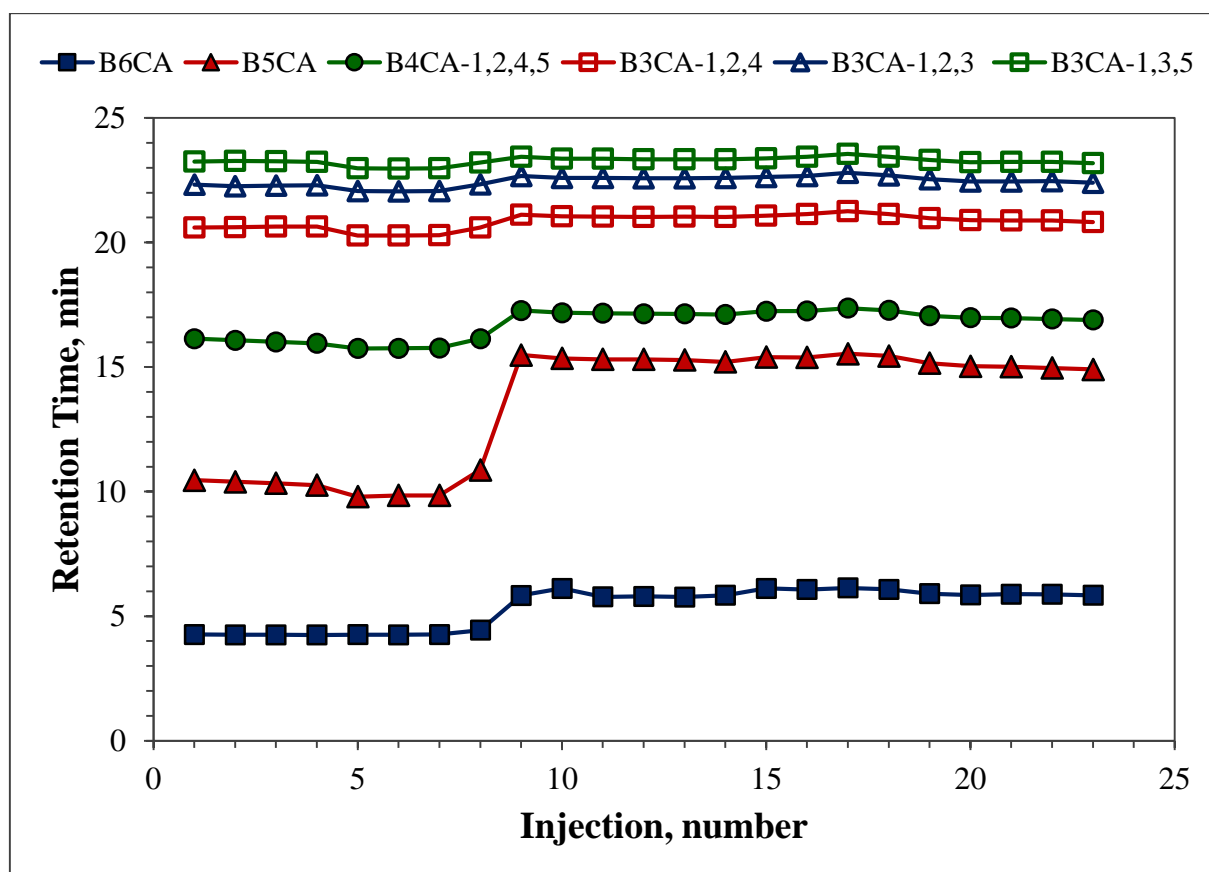
**Table 5.** Summary of BPCA analysis, acquired by HPLC.

Date of analysis	Compounds analyzed	Number of injections	Injection volume uL	Successful separation
2013-04-22	Individual, B3CA-1,2,3, B3CA-1,2,4, B3CA-1,3,5, B4CA-1,2,4,5 standards	8	10	No
2013-04-23	Individual, B3CA-1,2,3, B3CA-1,2,4, B3CA-1,3,5, B4CA-1,2,4,5 standards	8	10	No
2013-04-24	Individual, B3CA-1,2,3, B5CA	3	10	No
2013-04-29	Individual B3CA at 20, 200, 1000 ng; BPCA mix "200 ng" standards	6	10	Partly
2013-05-02	Individual B5CA, B6CA; BPCA mix 38 ng per standard	6	10	Partly
2013-05-07	Individual BPCA:s at 20, 80, 160, 200 ng; BPCA mix at 2, 4, 8 ng; standards	33	10	Yes
2013-05-13	Individual B4CA-1,2,4,5 at 20, 80, 160, 200 ng., standards	6	10	No
2013-05-15	BPCA mix "200 ng"; Samples Hexane1, Hexane2, Hexane3	5	10	Yes
2013-05-27	BPCA mix at 2, 4, 6, 8, 16 ng; Chernozem samples Daniel A5, A6	9	Varied	Yes
2013-06-01	BPCA mix at 2, 4, 6, 8, 16 ng; Rice Straw 1,2,3; Melanoidin 1,2,3; Chestnut Wood 1,2,3; Chernozem 1,2,3,4,5	25	Varied	No
2013-06-07	BPCA mix at 2, 4, 6, 8, 16 ng; Rice Straw 1,2,3; Melanoidin 1,2,3; Chestnut Wood 1,2,3; Chernozem 1,2,3,4,5	25	Varied	Yes
2013-06-19	BPCA mix at 50, 75, 100, 200 ng; Rice Straw 1,2,3; Melanoidin 1,2,3; Chestnut Wood 1,2,3; Chernozem 1,2,3,4,5; ACN	25	10	Yes

## 4.1. Peak identification and confirmation

### 4.1.1. Retention Time

To identify the correct retention time for the BPCA:s, each one of the standards were injected separately at the beginning. When approximate retention time was estimated, a mixture of BPCA standards was prepared and analyzed, good peak separation for BPCA standards was achieved directly as seen in Fig 2. The retention time for B6CA standard injection at the first successful test runs was at about 4,4 min, the retention time changed later, shifted to the right, or in other words increased by about one minute, as to be observed in the Figure 3 below. An unexpected “jump” of retention time for B5CA was also observed at the same time, with the shift from 10,9 min to 15,5 min, at the same time all other BPCA:s retention time also moved to the “right”, about 1-2 min for B6CA and 1,2,4,4-B4CA, and minor change for B3CA:s. This jump occurred at the same time when it was noted that the strength of orthophosphoric acid suddenly decreased. Afterwards the retention time for all the BPCA:s remained relatively uniform during the rest 15 injections. A minor decline of retention time for B5CA and 1,2,4,5-B4CA is observed for the last five injections.



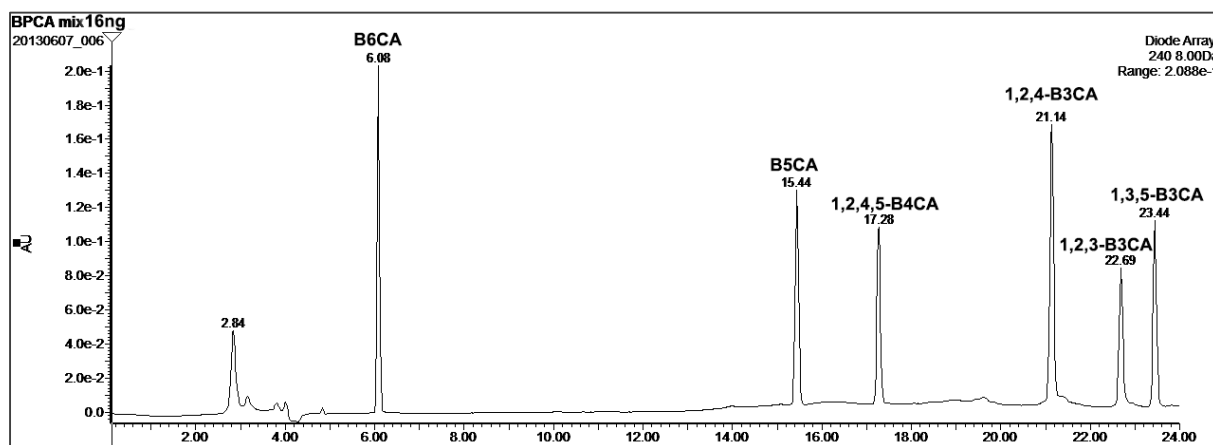
**Figure 4.** Retention times for different BPCA standards from 23 injections.

Composition of the mobile phases was the same, but it is believed that the pH in the orthophosphoric acid has changed, that combined with calibration problem for the pH-electrode for pH-measuring is believed to be the reason for the retention time shift. After the major shift to the right, the retention time stabilized for all the BPCA:s but still some minor alternation remained (Figure 4 and table 6). Most divergent retention time is for the B5CA, variation between earliest and latest retention time is 0,63 min, followed closely by B4CA,

1,2,3-B3CA, and 1,2,4-B3CA. Most stable retention times are for the B5CA and 1,3,5-B3CA, with variation of 0,36 and 0,37 min respectively. In overall minor changes in retention time could be a result of some minor changes of the solvents during the process due to evaporation, pH change or caused by gradient, those minor changes are considered normal (Snyder 1997).

**Table 6.** Table shows average, lowest, highest, and variation of retention times (min) for BPCA standards used for this study. Table shows values for all injections, left part, and values after the major retention time shift due to possible pH-change of the mobile phase. Variation columns show the difference between lowest and highest values.

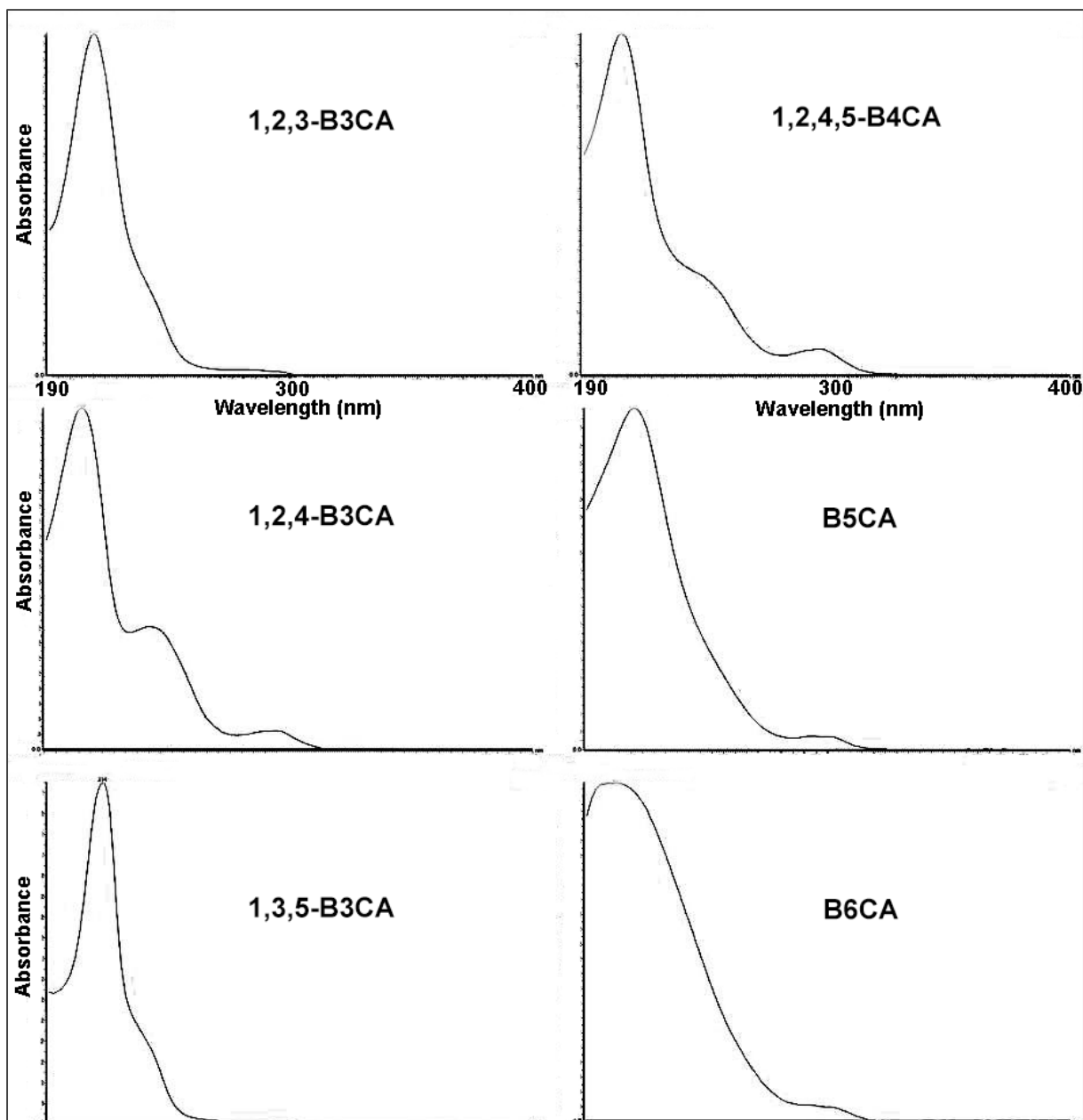
	Mean	Low	High	Variation	MEAN after shift	LOW after shift	HIGH after shift	Variation
<b>B6CA</b>	5,35	4,25	6,13	1,89	5,92	5,77	6,13	0,37
<b>B5CA</b>	13,50	9,80	15,53	5,74	15,25	14,91	15,53	0,63
<b>1,2,4,5-B4CA</b>	16,72	15,75	17,37	1,62	17,13	16,89	17,37	0,48
<b>1,2,4-B3CA</b>	20,84	20,28	21,25	0,97	21,02	20,82	21,25	0,43
<b>1,2,3-B3CA</b>	22,45	22,05	22,80	0,75	22,58	22,41	22,80	0,40
<b>1,3,5-B3CA</b>	23,27	22,97	23,55	0,58	23,34	23,19	23,55	0,36



**Figure 5.** An HPLC chromatogram of BPCA standards, B6CA at 5,82 min, B5CA at 14,91 min, 1,2,4,5-B4CA at 16,89 min, 1,2,4-B3CA at 20,82 min, 1,2,3-B3CA at 22,41 min, 1,3,5-B3CA at 23,17 min with injection volume 16 ng per BPCA. Absorbance at 240 nm. Chromatograph above show the peak are integration.

#### 4.1.2. Confirmation by spectrum figures

After the establishment of the retention times, specific spectrum figures of all six different BPCA:s were controlled. A graphic overview of the absorbance spectra for each individual BPCA used in this study is presented in Figure 5. The spectrum figures exhibited almost identical shape for the injections at different concentrations ranging from 2 to 16 ng. Although it was noted that the shape was somewhat poorer for the injections at lower concentrations, spectrums appeared to be noisier, with minor shape alterations. The combination of the retention time data and shape of the spectrum figures has been than used to identify the BPCA:s in the pyrogenic carbon reference material and chernozem samples.

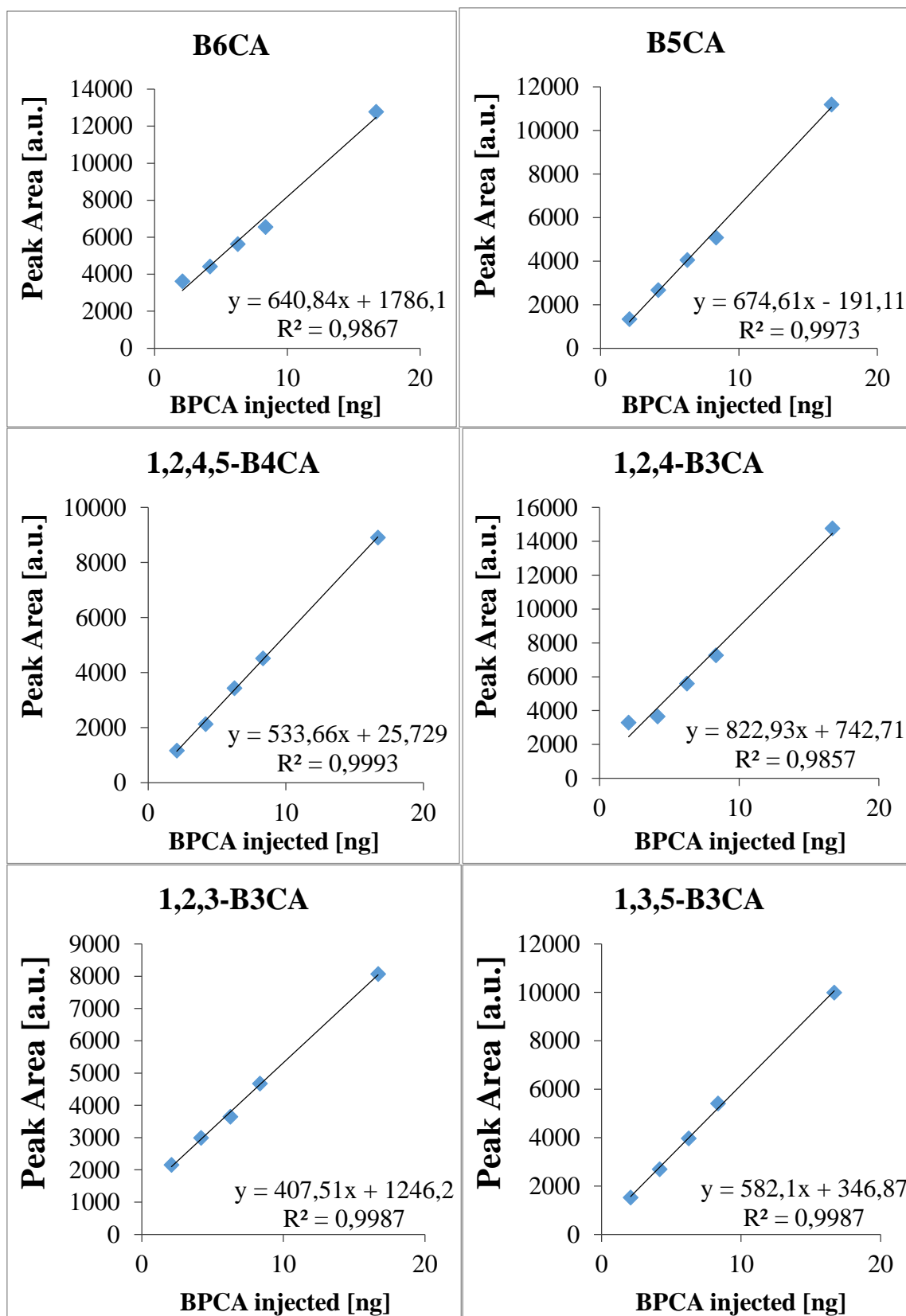


**Figure 6.** Graphs shows absorbance spectra (190-400 nm) figures of BPCA standards, acquired on Waters 996 PDA Detector, through MassLynx 4.1, based on the HPLC separation of BPCA standard mixtures with individual BPCA concentration of 16 ng per injection.

## 4.2. Calibration

### 4.2.1 Construction of calibration curves

Successful separation of the BPCA in the standard mixtures allowed the construction of calibration curves (Figure 7) for the quantification analysis of the chernozem and pyrogenic carbon reference materials.



**Figure 7.** A set of six function graphs showing the calibration curves based on the mean peak area (n=3) for the six BPCA standard signal plotted against injected concentration at 2, 4, 6, 8, and 16 ng (blue diamonds).

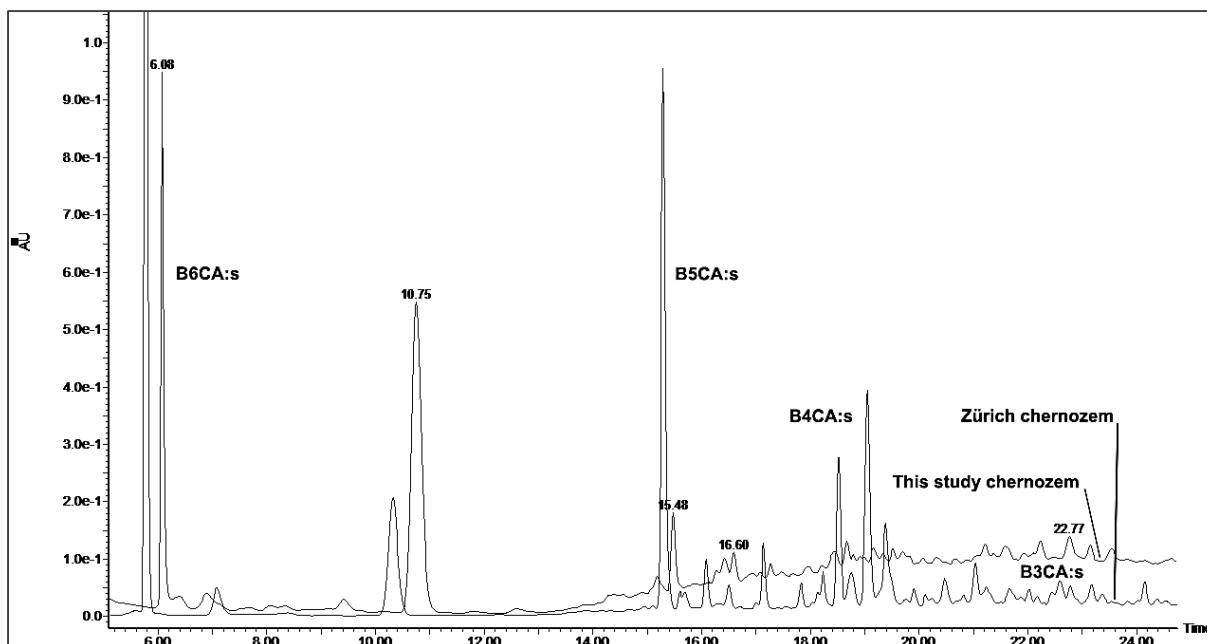
Square of correlation coefficient  $r^2$  is between 0,9905 (B6CA) and 0,999 (1,2,3-B3CA) for all three times calibration mixtures were analyzed, which indicates strong correlation. The calibration curve is based on 5 points. Injected amount of BPCA is about 2,1, 4,17, 6,23, 8,34, and 16,68 ng. The concentration below 2 ng is therefore very uncertain, the uncertainty increases the more far away from the calibration line the corresponding peak area is. The peak areas that are equal to concentrations below 2 ng are probably underestimated.

#### 4.2.2. Calibration range

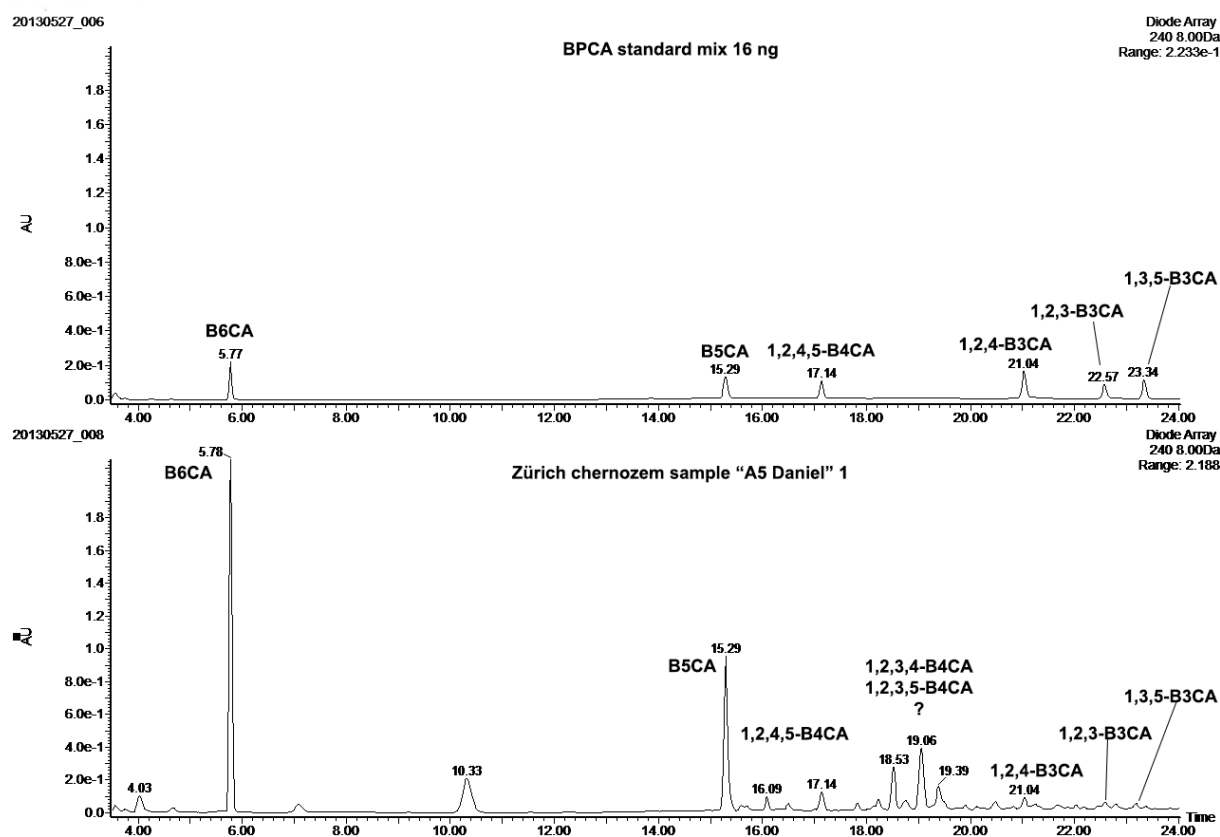
Different injection concentrations for the BPCA-standards have been tried out. Injection of 200 µg was a lot over the detection limit in the PDA, followed injection at 2 µg was also too high. For the correction, standards were diluted 100 times for the injection to a set of concentrations starting with 0,02 µg or 20 ng, with total set of concentrations of 10, 200 and 1000 ng. Concentration in the stock solutions for the BPCA:S was 20 000 µg ml<sup>-1</sup>. It was planned to use the BPCA standard mixtures with concentrations 25, 50, 75, 100, and 200 ng per BPCA, per injection. A misunderstanding during the preparation of the BPCA standard mixtures has led to lower individual concentration of BPCA, standards than expected. Instead of range 25, 50, 75, 100, and 200 ng the calibration range became 2,09, 4,17, 6,26, 8,34, and 16,68 ng (a mix of all sex BPCA, is equivalent to 25, 50, 75, 100, 200, the error). The same BPCA-standard mixtures, with low concentration range (2-16ng), were used during all the analysis.

#### 4.3 Sample analysis

As soon as all six standards were identified and the problems with the peak distortion were resolved, the analysis of the samples took place. Totally 5 batches were prepared for the BPCA-analysis, one batch with only hexane, one with only chernozem samples from Zürich and three large batches with pyrogenic carbon standard materials and chernozem samples prepared in Stockholm. The HPLC instrument malfunctioned during the analysis of one of three last batches and results are from this batch are not included in this study. Prepared hexane samples were the first ones to achieve good baseline separation of the peaks, with 3 replicates (Figure A3 in the appendix). The retention time for B6CA, 1,2,4,5-B4CA, B3CA-1,2,3 during the hexane analysis was about 1 min earlier (about 4,40 min) compared to later runs. The retention time for B5CA was much earlier, at about 10 min compared to later runs 15 min. At the same time a large peak at 5,8 min remained in the hexane separation, which firstly thought to be the B6CA, but the spectrum check revealed that the peak was something else. Later the chernozem soil samples from Zürich, labelled "Daniel A5" and "Daniel A6". Zürich soil samples were already pre-treated with digestion and filtering method and ready for the HPLC. The separation of peaks was good and generally matched to the graph presented by Wiedemeier et. al. 2013, (not shown here).



**Figure 8.** Chromatogram shows BPCA-separation of chernozem soil sample, a comparison between preparation by Wiedemeier at Zürich University and preparation at Stockholm University (This study chernozem). B6CA:s and B5CA:s clearly coincides in both samples, amount is much lower in Stockholm sample. Chernozem prepared at Stockholm was injected at 20  $\mu$ L injection, and Zürich chernozem was injected at 10  $\mu$ L.



**Figure 9.** Chromatogram above shows BPCA:s standard separation by HPLC, concentration at the injection is 16 ng per individual BPCA. Chromatogram below shows chernozem soil sample "Daniel A5" prepared in Zürich (Wiedemeier). X-axis is time in minutes. Y-axis is signal or absorbance unit. Two chromatograms are aligned to show the BPCA matches.

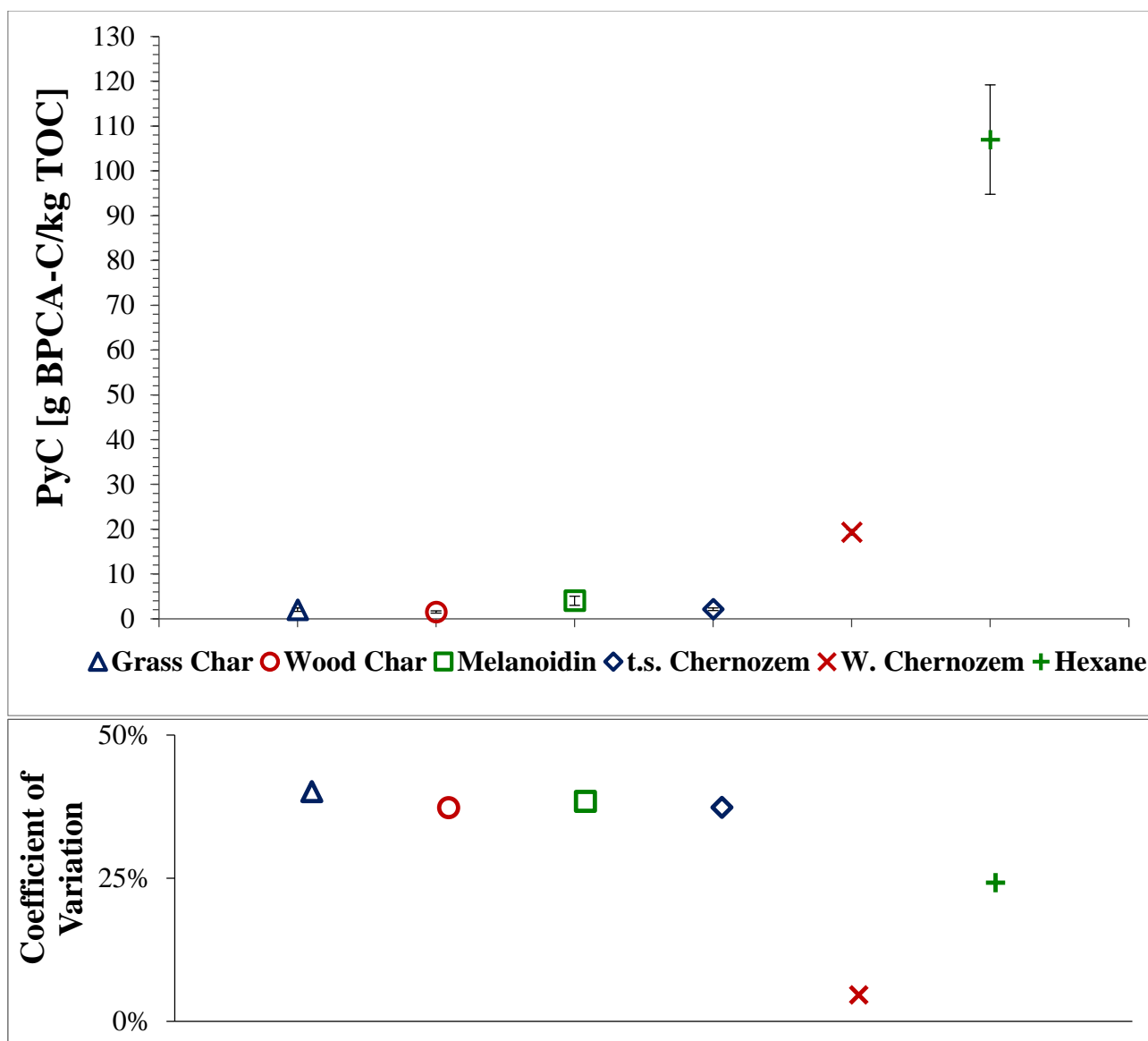
Figure 8, above, shows a chromatogram with comparison of the Wiedemeier chernozem soil sample with chernozem prepared at Stockholm University. The major difference between two samples is that the BPCA peaks are generally much higher for Wiedemeiers sample. The retention time seems to match quite well to BPCA-standard mixture injection, with some minor differences. It is hard to distinguish B3CA:s from each other in this chromatogram since they are too small. Both samples exhibit a large artefact peak at about 10,5-10,75 min, which is present in all other samples, the nature of which is unknown. Distinct B6CA peaks at about 6 min (except hexane) are observed for samples (appendix fig. 13-16). All other remaining BPCA:s (B5CA:s, B4CA:s, and B3CA:s) are rather very small or undistinguishable for all the samples. Large artefact peaks eluted at about 10.30 min, and minor unknown peaks at about 16 min, and around 18 min are present in chromatograms for all the samples (soot, char, melanoidin, chernozem, see appendix fig 13-16). Unknown peaks around 16 min could probably be the two remaining B4CA:s, but the confirmation is impossible without the corresponding standards. To calculate the remaining B4CA:s, it was assumed that the relative contribution of missing B4CA:s is about the same as for 1,2,4,5-B4CA, the amount of B4CA-1,2,4,5 was multiplied by two. Peaks for 1,2,3,4-B4CA and 1,2,3,5-B4CA couldn't have been identified in this study, but according to Wiedemeier, 2013, they are located in the range 18-20 min, as seen on Figure 9. However, the peak size of B4CA:s, those that are believed to be the remaining B4CA:s (peaks at 18,53 min, and 19,06 min) differs a bit, they are visually not equal.

#### 4.4. Concentration of Pyrogenic Carbon

In order to compare the quantification results from this work with the data from other studies, pyrogenic carbon content was calculated as g BPCA carbon per kg Total Organic Carbon (g BPCA-C/ kg TOC), concentrations of carbon pyrogenic (average concentration, error bars, and coefficient of variation) in g BPCA-C/kg-TOC are presented in Figure 10 and Table 7. The results were also calculated as relative values, as %-Pyrogenic carbon of TOC, since some of the writers have chosen to report pyrogenic concentration that way, the relative values are presented in Figure 11 and Table 7. For the detected BPCA amount and calculation of pyrogenic carbon content for all samples and their replicates see appendix figures A2-A6 (peak area data is not included).

**Table 7.** Pyrogenic carbon concentrations in chernozem prepared in Zürich (Wiedemeier), chernozem prepared at Stockholm University (Chernozem), hexane soot, lignocellulosic char derived from rice straw grass and chestnut wood, and melanoidin each reference material quantified by BPCA method (Wiedemeier, 2013).

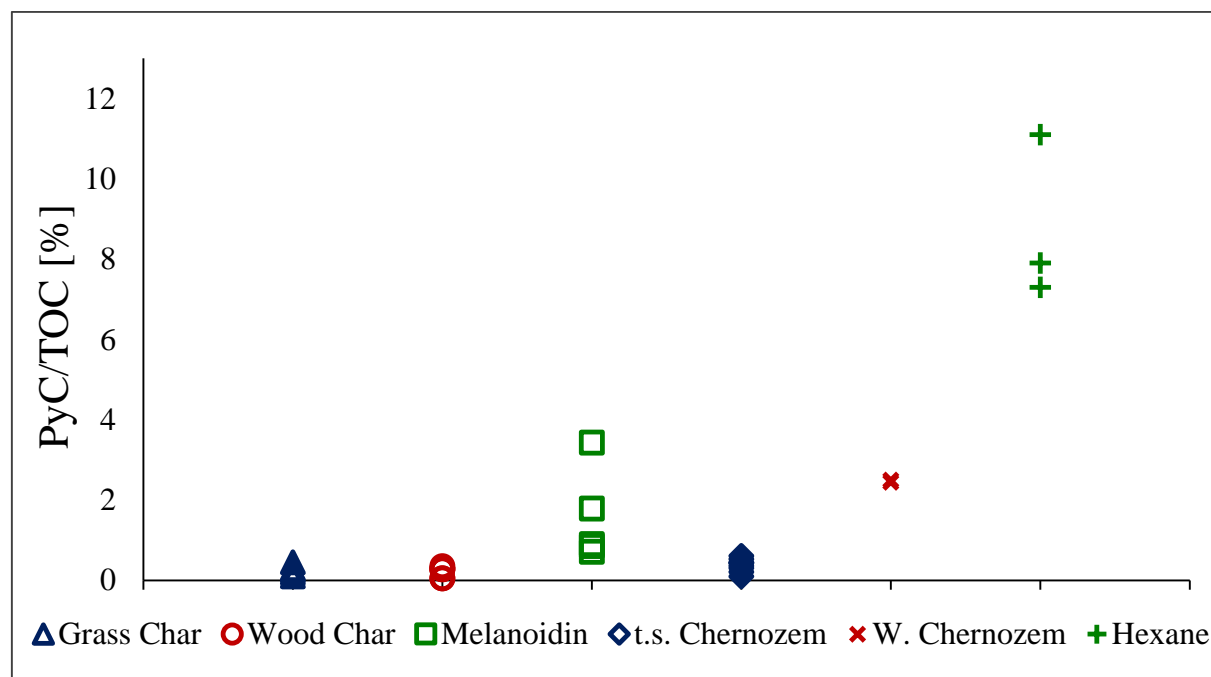
Sample	N of replicates	Average g BPCA kg <sup>-1</sup> TOC	Standard deviation, sample, g BPCA kg <sup>-1</sup> TOC	Min, g BPCA kg <sup>-1</sup> TOC	Max, g BPCA kg <sup>-1</sup> TOC	Coefficient of Variation	Average %-PyC of TOC
Wiedemeier	2	19,29	0,89	18,67	19,92	5%	2,47
Chernozem	9	2,13	0,79	0,84	3,19	37%	0,38
Hexane	3	106,97	25,92	87,99	136,50	24%	8,77
Grass char	5	2,02	0,81	0,84	3,00	40%	0,25
Wood char	6	1,51	0,56	0,71	2,07	37%	0,22
Melanoidin	5	4,01	1,54	2,87	6,58	38%	1,54



**Figure 10.** The graph above shows amount of pyrogenic carbon (PyC) measured by BPCA-method in reference materials grass char (n=5) wood char (n=6), melanoidin (n=5), this study chernozem, "t.s. Chernozem" (n=9), and chernozem prepared by D. Wiedemeier at University of Zürich, "W. Chernozem" (n=2). Pyrogenic Carbon (PyC) is expressed as a mass portion of the Total Organic Carbon and the mean values. Error bars are within markers for all samples except hexane. The adjacent graph below shows the coefficient of variation for the same samples and reference materials.

The highest amount of pyrogenic carbon was found in hexane samples (n=3), with a mean value of 106,7 g BPCA-C/kg TOC or at average of 8,77 % PyC/TOC, followed by chernozem soil sample prepared by Weidemeier (n=2), 19,3 g BPCA-C/kg TOC, or average 2,47% PyC/TOC. BPCA amount was detected in melanoidin at a mean concentration that is higher than in rice straw char, wood char and chernozem treated for this study. The average amount of pyrogenic carbon in melanoidin (n=5) was quantified to 4 g BPCA-C/kg TOC, or 1,54% PyC/TOC. The mean amount of pyrogenic carbon in chars was about the same, 1,5 g BPCA-C/kg TOC, or 0,22% PyC/TOC in wood char (n=6), respectively somewhat higher 2 g BPCA-C/kg TOC, or 0,25% PyC/TOC in rice straw grass char (n=5). Chernozem samples (n=9)

prepared at Stockholm University contained an average of 2,1 g BPCA-C/kg TOC, or 0,38% PyC/TOC. See table 10 below for specific statistical data. A number of accidents and errors during the laboratory work has led to an uneven amount of the sample replicates. Despite that a coefficient of variation were calculated to get an overall picture, and rough estimate of the analytical differences between different samples, fig 14 above. Highest coefficient variation is for the grass char, where it is at 40%; and in melanoidin at 38%; followed by wood char and chernozem prepared at Stockholm University, at 37 % respectively. Lower coefficient of variation was in hexane at 24%, and the lowest variation in two replicates of Wiedemeier chernozem, at only 5%.

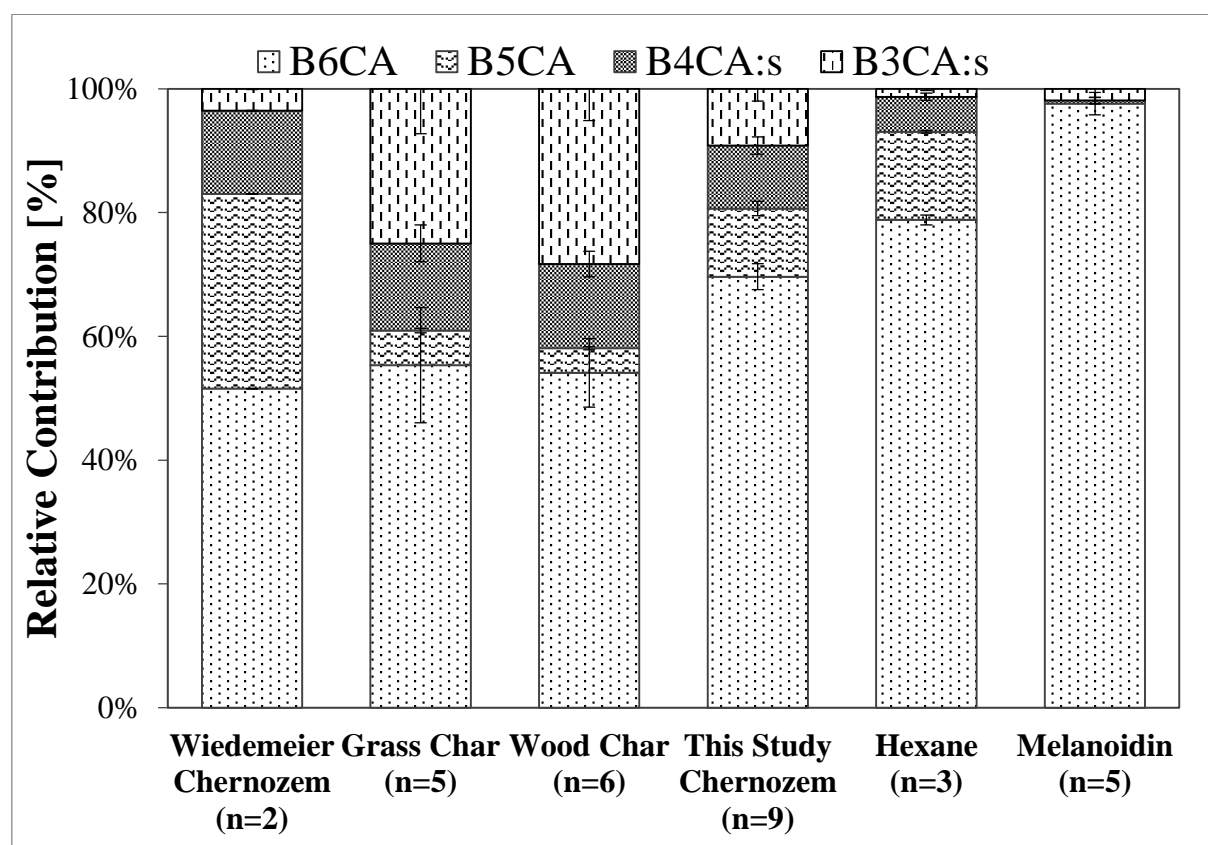


**Figure 11.** Pyrogenic carbon concentrations for each reference material, as proportion of TOC, detected by BPCA molecular method in this study. Grass char, n=5; wood char, n=6; melanoidin, n=5; this study (t.s.) chernozem, n=9, Wiedemeier chernozem, n=2, hexane, n=3.

#### 4.5. Contribution of BPCA:s

An increasing contribution of B6CA evidences the more condensed structure of pyrogenic carbon and subsequently higher or increasing combustion temperature (Glaser, 1998; Dittmar 2008). The contribution of the BPCA:s can also be used as an indicator of the size of spherule particles with aromatic structure. For a further estimation of the pyrogenic carbon quality, contribution of the different BPCA:s was calculated, and the results are plotted as a %-age histogram in Figure 12. Highest contribution of B6CA was found in hexane soot at mean value of 78,8 %. The B5CA contribution in hexane is 14% which is the second highest after Wiedemeier chernozem sample, 31,5 %. The B4CA:s contribution in hexane is slightly lower than in other reference materials, about 5,7%. Considerably lower amount of B3CA:s is found in hexane which is only 1,3% of the total amount of BPCA:s. The highest spread between different molecular BPCA markers is found in Wiedemeiers chernozem, where the half of the amount is B6CA, a one third consists of B5CA, on tenth B4CA:s and smaller amount of B3CA:s stays for 3%. Similar contribution is to be found in grass and wood char, where the amount of B6CA:s is little higher compared to Wiedemeiers chernozem, ranging between 54-55%, a slightly different contribution of B5CA, 4% for wood char and 5,5% for grass char,

with almost identical B4CA contribution at 13,6-14%. B3CA:s in the both chars are slightly more contributed than in the rest of the reference samples, ranging between 13,6-14%. Chernozem assayed in this study, has relatively high contribution of B6CA, about 69,6%, which is 20% higher than Wiedemeiers chernozem samples. The other BPCA:s molecular markers are evenly distributed at about 10 % in this study chernozem. Pyrogenic carbon in melanoidin consists of exclusively B6CA, 97,6%, no B5CA was detected in any replicate, and very small amounts of B4CA:s and B3CA:s were detected at 0,5% and 1,9% respectively. In general the proportion of B6CA was significantly higher than other BPCA:s. B6CA/B3CA ratio is used to describe aromaticity of material. The highest B6CA/B3CA ratio was found in hexane, about 62, and the second highest ratio was found in chernozem prepared by Wiedemeier, a value of almost 15. Significantly lower B6CA/B3CA ratios are found in char samples (about 2) and this study prepared chernozem (about 8). The contribution of individual BPCA:s is slightly different in chernozem sample prepared for this study compared to Wiedemeier chernozem. Higher amounts of B6CA, and B3CA are detected in this study chernozem, about 70%, and 9% for B6CA and B3CA respectively; while Wiedemeier chernozem yields about 52% B6CA and 3,5% B3CA. At the same time higher contribution of B5CA is detected in Wiedemeiers chernozem, 31%, compared to only 11% B5CA in this study chernozem. Relative contribution of B4CA is about the same in both chernozem samples, about 10% in this study, and somewhat higher, 13% in Wiedemeier chernozem.



**Figure 2.** The histogram shows relative contribution of each individual BPCA:s in chernozem sample from Zürich (Wiedemeier) and chernozem average of different injection volumes (5, 10, 15, 20 and 25 ng) prepared at Stockholm University; hexane soot, rice straw char, chestnut wood char, and melanoidin. Contribution and y-error bars for different samples are based on the average between each BPCA:s replicates.

## 4.6. Blanks

It was considered to use any measured concentration of BPCA:s in blanks as detection limit. Anyhow, only B6CA was detected in some of the BPCA:s, at very low concentrations, about 1 ng per injection. If the B6CA was detected in a separation previously the analyses of the samples, the amount detected B6CA was then withdrawn from following analysis in order to correct the amount with respect to the possible un-retained rests from previous runs.

## 5. Discussion

### 5.1. Comparison to other studies

Pyrogenic carbon quantification has been suffering of the unacceptable result variation. The results from this study has been compared with a number of other studies that has applies BPCA method. Comparison to several other studies that applied BPCA-method for quantification of the pyrogenic carbon content in black carbon reference materials are presented in the Table 8, and discussed further below.

**Table 8** Average concentrations (g BPCA-C/kg TOC) of the pyrogenic carbon in different black carbon reference materials, using BPCA-method; applying HPLC for this method, Wiedemeier (2013), Schneider (2010); and GC for Hammes (2007), Brodowski, (2005b). Dash marks, “-“, means that the material was not included in the given study (no data). <sup>1</sup> chernozem prepared in this study/chernozem prepared by Wiedemeier.

Reference materials	This study	Wiedemeier (2013)	Schneider (2010)	Hammes (2007)	Brodowski (2005)
Chernozem	2,13/19,29 <sup>1</sup>	105	66,2	3,8	19,8
Grass char	2,02	165	212	155	-
Wood char	1,51	180	242	183	-
Hexane	106,97	230	-	240	-
Melanoidin	4,01	-	-	5	13,03

#### 5.1.1. Chernozem

Hammes et. al. (2007), reports the concentration of 3,8 g kg<sup>-1</sup> TOC of BC in chernozem reference material. Schneider et. al. (2010), quantified BPCA carbon in chernozem at a mean value of 66,2 g BPCA-C/kg organic matter (OC), with relatively large scatter of the data with minimum value of 51,1 BPCA-C/kg OC and the maximum of 81,4 g BPCA-C/kg OC (obtained by 16 measurements, with n=3 replicates). Brodowski et. al. (2005b), reports BPCA-C at about 19,8 kg<sup>-1</sup> C which is in agreement with the pyrogenic carbon detected in the chernozem soil sample prepared by Wiedemeier, at the mean value of 19,29 g BPCA-C kg<sup>-1</sup> TOC (Table 8). However, Wiedemeier et. al. (2013), analyzing the same soil sample reported much higher value of about 105 g BPCA-C kg<sup>-1</sup> TOC (value is not precise, derived from a diagram). Lower concentration of the PyC in chernozem, in this study, could indicate 1) possible underestimation in this study due to low calibration range (2-16 ng injected); 2) underestimation related to the possible issues with the HPLC-system and or detector. Chernozem soils sample prepared for this study, averages at 2,13 g BPCA-C kg<sup>-1</sup> TOC, is almost two orders of magnitude lower than the content of pyrogenic carbon in chernozem by Wiedemeier, et. al. (2013). The discrepancy strongly indicates a possible insufficient oxidation of soil samples, since exactly the same method has been reproduced in this study. Possible loss of the BPCA during the handling is another possible explanation, which is difficult to verify since no internal standard has been used.

### 5.1.2. Chars

BPCA method (both GC and HPLC) yield significantly higher amounts of pyrogenic carbon in all other studies. In the study of charcoals prepared at different temperatures by Schneider, 2010, the pyrogenic carbon content in wood char pyrolyzed at 450°C, is reported at about 212 g BPCA-C kg<sup>-1</sup> TOC, and 242 g BPCA-C/kg TOC charcoal for wood char. Those concentrations are almost 8 orders of magnitude higher than found in this study. Even greater amount of BPCA carbon in char is reported by Roth, 2012, the value is 402 g black carbon (BC)/kg OM (BPCA-C is referred as BC), however the char was produced at much higher temperature of 800°C. Both studies applied GC for the separation of BPCA:s. Hammes et. al. (2007), reports a mean value of 183 g kg<sup>-1</sup> TOC in the wood char, and 155 g kg<sup>-1</sup> TOC in grass char, applying BPCA method, also significantly higher amounts compared to this study. Results for pyrogenic carbon (BC) in chars and soot, analyzed with BPCA method by different laboratories evaluated in ring trial by Hammes et. al. (2007), ranges up to a factor of three, with a mean value reported at 26% PyC/OC (BC/OC), compared to 0,22-0,25 %-PyC of TOC measured in this study. Wiedemeier, 2013 reports values 165 g BPCA-C/kg for grass char, and 180 g BPCA-C/kg for wood char, values that exceeds concentrations reported in this study by a factor of 82 (grass) and of 119 (wood). What is more surprising is that the mean concentration of PyC in wood char is lower than in grass char, which contradicts the results from all other studies, where the common trend is to measure slightly higher amounts of PyC in wood chars due to expected higher content of lignin which contributes to higher amount of BPCA:s.

### 5.1.3. Hexane soot

Ring trial by Hammes et. al. (2007), reports concentration of pyrogenic carbon as proportion of organic carbon (OC), with highest amount of 50% of OC, with lowest value of 5% PyC of OC, with an average of 20% PyC of OC, with 3 replicates for hexane, average value of 240 g BPCA-C/kg TOC. The highest proportion of PyC measured in hexane in this study is 11,1%, the lowest is 7,3%, and the average is 8,77%. Since data reported by Hammes et al (2007) scatters a lot, it is difficult to compare to the results from this study, however the lowest measured values in this study seems to be somewhat in agreement with Hammes, et. al. 2007. Wiedemeier, et. al. (2013), measures concentration of PyC in hexane soot at about 220 g BPCA-C/kg TOC with very small variation (n=3), compared to 88 g BPCA-C/kg, the lowest measured in this study, and 136,5 g BPCA-C/kg the highest, and average at 106,97 g BPCA-C/kg. Again the values are somewhat close to each other. At least the discrepancy is much lower than compared to other materials analyzed in this study. The hexane was the first material to be assed, and it was digested by fresh HNO<sub>3</sub> that was purchased just days before the sample preparation started. All other materials has been digested with an older HNO<sub>3</sub>, no quality control was made on the older acid. Those facts strongly suggest that the large discrepancy of the results and low PyC concentration measured in chars and chernozem could be a result of incomplete oxidation.

### 5.1.4. Melanoidin

Brodowski et. al. (2005b), concludes that the earlier developed BPCA method by Glaser (1998), which uses HCl 32 % pretreatment, in addition to HNO<sub>3</sub> digestion has led to BPCA formation in non-BC containing materials such as melanoidin. A mean value of 13,03 g BPCA-C/kg TOC, generated by melanoidin using BPCA method, was reported by Brodowski et. al. (2005b). Mainly B4CA:s and B5CA:s were detected, which is different compared to the

results in this study, because melanoidin generated almost exclusively B6CA:s and some uncertain amount of B3CA:s. Brodowski et. al. (2005b), also concluded that BPCA contribution from melanoidin found in soils is not significant, but the pretreatment with HCl should be replaced by TFA, since TFA do not lead to the formation of the BPCA. In the study by Wiedemeier 2013, melanoidin was not included in the assessment. Hammes et. al. (2007), ring trial reports a mean value of 5 g kg<sup>-1</sup> in melanoidin, applying BPCA method, which is close to the outcome in this study, with average amount of 4 g BPCA-C/kg TOC in melanoidin.

#### 5.1.5. BPCA contribution

Dittmar et. al. (2008), reports the highest proportion of B6CA, at 70% of total BPCA in analyzed activated charcoal. B5CA was detected at 12% in the same study. Two B4CA:s, 1,2,3,4-B4CA and 1,2,4,5-B4CA were reported at 18% totally. Whereof 1,2,4,5-B4CA was detected at only 1%. Such low concentration of one individual B4CA contradicts the assumption to compensate for the commercially unavailable B4CA:s by multiplying the analyzed 1,2,4,5-B4CA by two, as it was done in this study. At the same time no B3CA was detected at all in charcoal sample by Dittmar et. al. (2008), which makes the sensitivity of the results questionable. Dittmar et. al. (2008), used pyrene (polycyclic aromatic hydrocarbon with 5 benzene rings, similar material to hexane) as a highly condensed reference material, the contribution of B6CA was reported at 81%, which is similar to the hexane B6CA proportion at 78,81 % in this study. High yield of 1,2,3-B3CA, at 19 % was detected in pyrene by Dittmar et. al. (2008), while in this study only about 1,28 % for all B3CA:s was detected in hexane, furthermore an uncertain number since the low concentrations of B3CA, and unreliable detection limit. Roth et. al. (2012), reports higher contribution of B6CA for the hexane sot, at about 75 % of total BPCA contribution, quite high proportion, almost in line with B6CA in hexane for this study (78,81 %, B6CA). In the same study Roth et. al. (2012) notes that the proportion of B4CA:s is higher in the grass and wood char standard materials that has been charred at 800 °C, ranging between about 30% for wood and to 38% for grass char respectively. In this study, almost exclusively B6CA, about 98 %, was found in melanoidin samples. The founding differs from the results achieved by Brodowski et. al. (2005b), authors reports a more even distribution of individual BPCA:s, ranging between only 12% of B6CA, and 40% of B4CA in melanoidin. Charred plant material analyzed by Brodowski et. al. (2005b), has also demonstrated a much more even distribution of BPCA:s compared to this study, with all BPCA:s contributed at around 30 %, except B3CA which was less abundant at about 12%. Highest relative proportion of B6CA was reported in the chernozem soil sample by Brodowski, et. al. (2005b), at about 35%, compared to 52% B6CA measured in Wiedemeier chernozem sample, and 70% B6CA in chernozem prepared in this study. Comparing to results by Brodowski et. al. (2005b), significantly lower contribution of B5CA was detected in chernozem prepared for this study, 30% B5CA by Brodowski, et. al. (2005b), and 11% for this study; on the other hand chernozem prepared by Wiedemier had about the same yield of B5CA at 31%. B4CA:s contribution in chernozem by Brodowski et. al. (2005b), was reported at about 25%, which is higher by almost a factor of 3, compared to B4CA in chernozem detected in this study, both for Wiedemeier et. al. (2013), and this study chernozem (10% and 13% respectively), and with lower B3CA contribution at about 5%. A relatively high yields B3CA:s, at about 25-28 % in chars was found in this study, compared to 10% B3CA:s in char samples by Brodowski et. al. (2005b). But B3CA:s yields are almost in line with B3CA contribution in chars (charred at different temperatures) reported by Glaser

(1998), where the B3CA contribution ranges between 20-25%. However the B3CA detection was highly uncertain which makes it hard to do any further conclusions. According to the study performed by Schneider et. al. (2010), the B6CA contribution in the chernozem sample was variable, 32-50%, with typical values at 40-45%, with lowest occurring B3CA at 1 to 10%. For the chestnut wood charred at 450 °C by Schneider et. al. (2010), the contribution of the individual BPCA:s is following: B6CA about 32%, B5CA at about 35%, B4CA:s at about 26%, B3CA:s at about 5% (values are not precise, since they are derived from a diagram). Even contribution of B5CA and B6CA is slightly different from the contribution of those BPCA:s in this study, where B6CA is much higher, about 55% for both chars. The amounts of B5CA and B4CA:s in chars are significantly lower in this study compared to Schneider et. al. (2010), and contribution of B3CA:s is much higher, up to a factor of six. Since it is believed that oxidation was incomplete for some samples during the digestion, and due to possible underestimation of some of BPCA:s, those that were out of the calibration range it is difficult to compare the contribution to the results from other studies. However n-hexane exhibits the most similar contribution comparing to other results.

## 5.2. Method improvement

One of the goals with this study was to achieve reproducible results compared to the developers (Wiedemeier, 2013) of this molecular marker method. Due to the unexpected results after multiple attempts to apply BPCA-method, questions regarding pre-treatment method arose, since the HPLC-ready samples from Wiedemeier differ so much from those prepared at Stockholm University, and for the overall discrepancies described above.

### 5.2.1. Sample preparation

Fresh nitric acid (65%) disappeared from the lab. An older flask with nitric acid was used. Perhaps the older nitric acid was deteriorated, resulting in not enough oxidative strength to separate BPCA:s efficiently enough. Consequently the quality of the nitric acid (if not fresh) should be checked by titration to determine molar concentration of the acid, before it is used for the analysis. Hammes et. al.. (2007) suggests that loss of material due to the filtration during the pretreatment is possibly a contributor to the underestimated results. Method by Wiedemeier et. al. (2013) used in this study excludes this pretreatment step, but still it is possible that some of the pyrogenic material could have been lost by necessary filtration after the digestion, although the same filtration step is carried out by all other authors applying BPCA molecular marker method, which doesn't explain such great difference, for all materials assed in this study, except hexane. No internal standard has been added during the analysis of the BPCA standards to be able to trace the possible loss of pyrogenic carbon during the sample preparation, since the results are much lower than compared to other studies, perhaps for further studies the use of internal standards should be considered. Hammes et. al. (2008), points out that duration of derivatization, the temperature and also time after derivatization could affect the results significantly. But since all the samples were treated in exactly the same way, this theory wouldn't give a satisfying explanation for the discrepancies of the results.

### 5.2.2. Filtration

During the analysis it was noticed that the cellulose-filter started to decompose in the presence of HNO<sub>3</sub>. The question is if BPCA-similar molecules could be formed from the structure of the dissolved cellulose under the influence of the strong nitric acid. Freudenberg et. al. (1965) has reported the formation of BPCA:s after treatment of the organic matter with

strong acid. This artefact BPCA formation needs further investigation in future. The amount of BPCA that could possibly be formed, and more details on the reactions that result in the formation of artefact BPCA are needed to verify the influence on quantification results. Perhaps cellulose filter should be replaced by filter made of inorganic material, such as silica-based filter. The color of the solution after the resin columns was altered from transparent to lightly yellowish, which means some of the resin has probably leaking in to the samples during washing.

### 5.2.3. Drying of samples

Freeze-drying was carried out at 10°C. Samples were frozen with liquid nitrogen one by one and already frozen samples were kept in refrigerator to prevent them from melting. Samples were not dry at all after freeze drying at ORPM, 10°C, at 1 mbar system pressure over night. Solution of HNO<sub>3</sub>/H<sub>2</sub>O in the samples is corrosive for the centrifuge sample drum. Probably due to a leak in the freeze-dryer system or malfunctioning vacuum pump the drying process of the samples took too long time. It is under question if the problems with freeze-drying could affect the concentration of the BPCA, in such case there is perhaps a risk of BPCA:s being evaporated as a result of freeze-drying that took far longer time than usually is required for a standard procedure. Glaser (1985) describes that longer exposure (<8h) of the BPCA solutions to strong acid will eventually result in the decomposition of the BPCA-molecules, which will lead to the underestimation of the pyrogenic carbon. It is possible that the underestimation of the BPCA in this this study may have been a result of too long freeze-drying, since it was exposed to strong nitric acid for a much longer time than planned.

### 5.2.4. Measurement of pH

While preparing the mobile phase 1 for the HPLC separation of the BPCA molecular markers, the pH-meter was malfunctioning, and calibration could not be performed properly, or was inadequate for correct pH-measurement. For the second control of pH litmus paper was used, which is not precise enough as pH-electrode. Due to the problems with pH measurement, pH of the mobile phase fluctuated during the analysis. Variation of pH has affected the retention time for different BPCA:s. However, in overall the fluctuation of the retention times was within acceptable range to perform a successful separation, identification and analysis of the results.

### 5.2.5. Calibration Range

Unfortunately the BPCA standard mixture used for the calibration curve prepared incorrectly due to a misunderstanding, with wrong calibration range 2-16 ng instead of 20-200ng. Error was detected after the analysis, when the results were calculated. Anyway, this error majorly affected mostly the hexane, Wiedemeier chernozem samples, and some of the B6CA:s in other samples, since the concentration of B6CA was almost exclusively higher than all other BPCA:s, and therefore out of range of the calibration curve. The underestimation of the BPCA amount is expected if the measured peaks are outside, and above the calibration range (2-16 ng), since the actual relationship between peak area, and corresponded concentration is not linear. The actual curve for the function of peak area with respect to concentration is expected to flat out at higher concentrations, resulting in higher actual concentrations than calculated by calibration curve. Almost all BPCA:s in the hexane were detected above the calibration range. B6CA:s and B5CA:s are above the calibration range in Wiedemeier chernozem samples. Smaller volumes (5-10 µL) injections of the chernozem samples have significantly higher BPCA values that are below the calibration range. For the chernozem

injection at volumes 10-20  $\mu\text{L}$  the amount of detected BPCA:s is little higher and within the range for most of the samples and BPCA:s. Somehow, the amount of B3CA:s detected in the chernozem at 20-25  $\mu\text{L}$  injections is below the calibration range ( $<2\text{ng}$ ). The amount of the BPCA:s detected in rice straw, grass chars, and wood char is dominantly below the calibration range, except for B6CA, which is within range for mostly all the samples, except for one grass char replicate, and one chestnut wood replicate, where the B6CA signal is above the calibration range. One rice straw sample/injection has all BPCA:s signals, except for B5CA, within the calibration range. B6CA detected in the melanoidin is above the calibration range, and therefore is expected to be underestimated; 1,2,4,5-B4CA is detected within range in one replicate of melanoidin; two of the B3CA:s are detected above the calibration range in one melanoidin replicate. See Table A1 in the appendices section for all deviated results.

### 5.3. Future work with BPCA molecular marker method

The reproducibility of the results from different laboratories needs to be improved, and the more work needs to be done on the standardization of the method; there is still an ongoing discussion about the reliability of the method and use of different detection instruments. It is also expected that the BPCA molecular marker method tends to detect lower concentration of the pyrogenic carbon comparing to other detection methods, which means that there is a possibility that the BPCA method is not able to detect the most extensive and condensed pyrogenic carbon molecules (Hammes, 2007). During the sample preparation, when the sample is digested with  $\text{HNO}_3$ , some of the carbon may be lost by the conversion in to  $\text{CO}_2$ . To take that loss into account the use of a conversion factor is recommended by different writers. Schneider, (2010) concludes that it in overall it is better to not use any conversion factors at all to avoid erroneous quantification. But the fact that carbon loss during acid digestion remains, and need to be dealt with in future in order to reach the consensus in between different analytical methods for quantification of pyrogenic or black carbon. Perhaps a set of different conversion factors, adjusted to different materials, must be evaluated for compensation of the BPCA loss. In this study the filtration of the samples has been performed with three different steps, and it would be crucial to estimate the eventual loss of the BPCA:s during those steps, by using an internal standard. For the future assessment of the pyrogenic carbon, the questions about the use of internal standards should be an object of investigation. To properly calculate the limit of detection the more proper statistical approach is needed and therefore more standardized routines needs to be applied for establishment of the analysis routines.

## 7. Conclusions

The purpose of this study was to set up the laboratory instrumentation and apply a new BPCA-HPLC molecular marker method for quantitative and qualitative assessment of the pyrogenic carbon content in a set of environmental samples. It was initially planned to analyze pyrogenic carbon content in the sediment samples obtained from a lake in Thailand, used for climate analysis in another study, this goal was abandoned later since the BPCA method did not worked out well, despite several attempts to perform the analysis. Pyrogenic carbon is defined as carbonaceous material derived from combustion of organic matter, is thermally and chemically relatively stable, and resistant to biological degradation. Molecular marker method by Wiedemeier has been developed recently and it seems that it needs to be further evaluated, since the pyrogenic carbon results in this study have not reached the same range as in the precursor literature. Pyrogenic carbon in the chernozemic soil samples was

detected at much lower concentrations compared to recent studies, but the measured content is somewhat closer to older studies that applied gas chromatography for separation of the BPCA molecules. Unexpectedly low concentrations of the pyrogenic carbon were detected in pyrogenic carbon rich, positive control materials, such as grass chars and wood chars. The pyrogenic carbon content in chars is up to 8 orders of magnitude lower compared to some other studies. Incomplete derivatization, destruction of BPCA molecules during longer exposure to the nitric acid during the freeze-drying and preparation of the samples, are possible explanations to the low concentrations of pyrogenic carbon detected in chars. Amount of pyrogenic carbon in hexane soot was more in line with other studies, but the results suffered from underestimation, since the concentration calculation was based on the calibration curve that was out of range for the amount BPCA:s in the sample. However, successful separation on the HPLC instrument was achieved for all the samples. Surprisingly, chernozem soil sample prepared by another laboratory (ready for HPLC-injection solution), analyzed for comparison in this study, yielded five times lower PyC concentration compared to reported results from Zürich laboratory, which is also explained by the calibration range that is out of range. Pyrogenic carbon was detected in relatively low concentrations, in negative control material, melanoidin, that should not contain any carbon of pyrogenic origin. This finding confirms the theory of artificial BPCA formation in the melanoidin under the exposure to strong acid as reported in other studies, and makes the use of melanoidin as a negative control questionable. Lower contribution of B6CA:s in wood char compared to grass chars was detected, which contradicts the results from all other studies, where the B6CA amount is higher in wood chars since higher content of lignin contributing to more condensed structure in wood chars. Unreliable results of the PyC amount makes it difficult to compare the contribution of the BPCA:s to other studies, and make any further conclusions about the qualitative assessment of samples. For the future, the BPCA method needs further evaluation and development. Attention should be paid especially to different steps during the preparation of the samples, such as filtration, and freeze-drying, where small changes in duration and procedure conditions, are believed to drastically alter the outcome of results. Standardized use of the internal standard must be implied to control the loss of BPCA during many different handling steps. Since the derivatization of BPCA:s is not complete, a use of a conversion factor that is adjusted to different material should be more investigated. Other goals for the next workers should be to continue the improvement of the method applying more statistical approach in order to achieve improved reproducibility of the results.

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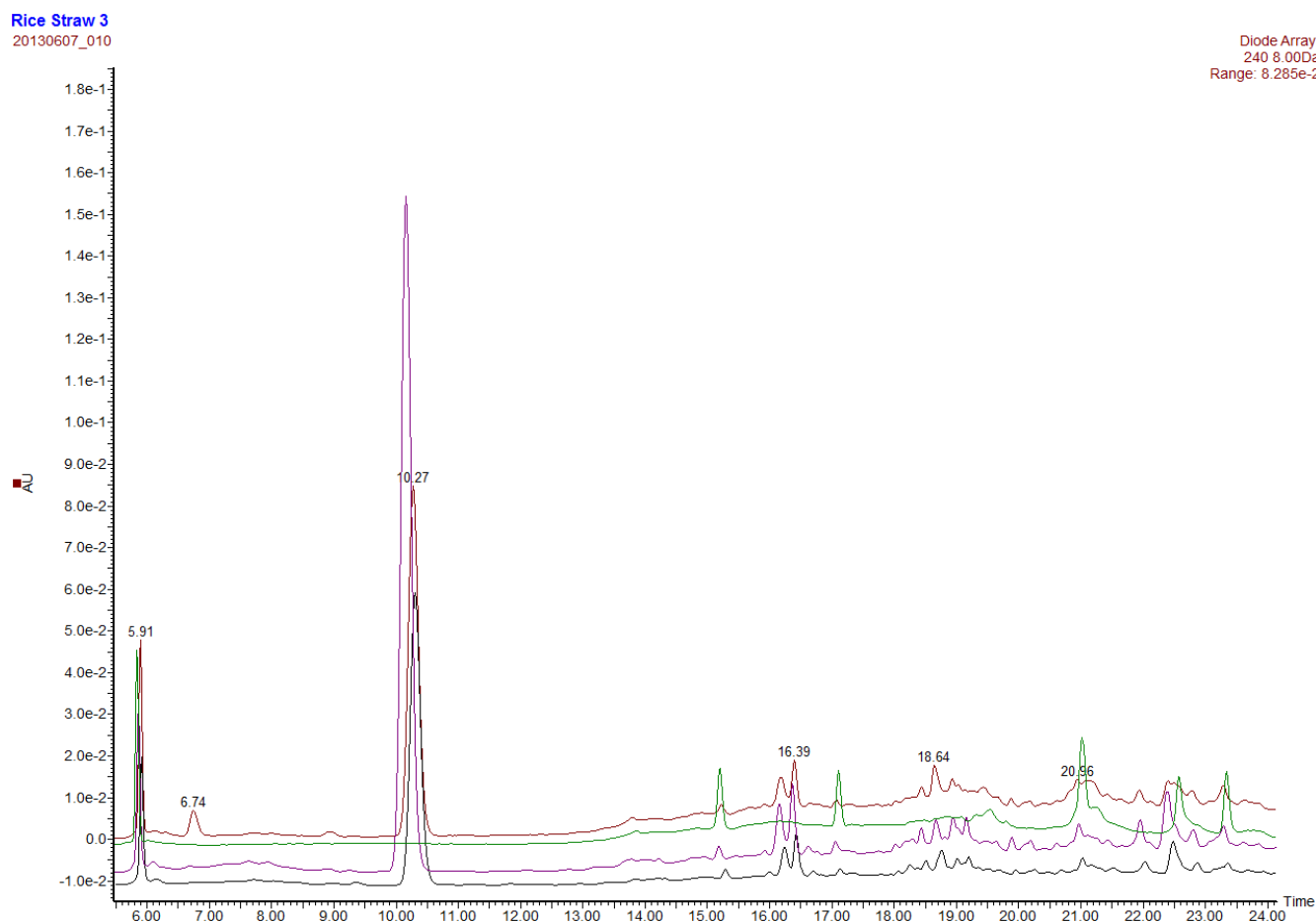
## 9. References

- Andreae, M.O., and Gelencsér, A., (2006), Black carbon or brown carbon? The Nature of light-absorbing carbonaceous aerosols, *Atmos. Chem. Phys.*, 6, pp 3131-3148.
- Benzig-Purdie, L., and Ripmeester, J. A., (1983), Melanoidins and soil organic matter - Evidence of strong similarities revealed by C-13 CP-MAS NMR, *Soil Sci. Soc. Am. J.*, 47(1), pp 56-61
- Bond, T.C. and Sun, H., (2005), Can reducing black carbon emissions counteract global warming? *Environ. Sci. Technol.*, 39, pp. 5921-5926.
- Bond, T. C., Doherty, S. J., Fahey, D. W., Forster, P. M., Bernsten, T., DeAngelgo, B. J., Flanner, M. G., Ghan, S., Kärcher, B., Koch, D., Kinne, S., Kondo, Y., Quinn, P. K., Sarofim, M. C., Schultz, M. G., Schulz, M., Venkayarman, C., Zhang, H., Zhang, S., Bellouin, N., Guttikunda, S. K., Hopke, P. K., Jacobson, M. Z., Kaiser, J. W., Klimount, Z., Lohmann, U., Schwarz, J. P., Shindell, D., Storelvmo, T., Warren, S. G., Zender, C. S., (2013), Bounding the role of black carbon in the climate system: A scientific assessment. *American Geophysical Union*, pp. 13-25.
- Brodowski, S., Amelung, W., Haumaier, L., Abetz, C., Zech, W., (2005a), Morphological and chemical properties of black carbon in physical fractions as revealed by scanning electron microscopy and energy-dispersive X-ray spectroscopy, *Geoderma*, 128, pp. 116-129.
- Brodowski, S., Rodionov, A., Haumaier, L., Glaser, B., Amelung, W., (2005b), Revised black carbon assesment using benzene polycarboxylic acids, *Organic Geochemistry* 36, p. 1299-1310 (Melanoidin at p. 1306).
- Dai, X., Boutton, T.W., Glaser, B., Ansley, R.J., Zech, W., (2005), Black carbon in a temperate mixed-grass savanna. *Soil. Biol. Biochem.*, 37, pp. 1879-1881.
- Dickens, A.F., Gelinas, Y., Hedges J.I., (2004) Physical separation of combustion and rock sources of graphitic black carbon in sediments, *Mar. Chem.*, 92, pp. 215-223.
- Dickhut, R.M., Canuel, E.A., Gustafson, K.E., Liu, K., Arzayus, K.Y., Walker, S.E., Edgecombe, G., Gaylor M.O., MacDonald E.H., (2000), Automotive Sources of carcinogenic polycyclic aromatic hydrocarbons associated with particulate matter in the Chesapeake Bay Region. *Environ. Sci. Technol.*, 34, p. 4635.
- Dittmar, T., (2008), The molecular level determination of black carbon in marine dissolved organic matter, *Organic Geochemistry* 39, pp. 396-407.
- Eby, G.N., (2004), *Principles of Environmental Geochemistry*. Brooks/Cole, 1<sup>st</sup> Edition, ISBN-13: 978-0122290619
- Elmqvist, M., Cornelissen, G., Kukulska, Z., Gustafsson, Ö., (2006), Distinct oxidative stabilities of char versus soot black carbon: Implications for quantification and environmental recalcitrance. *Global Biogeochem. Cycle.* 20, doi:10.1029/2005GB002629.
- Fernandes, M., B., Skjemstad, J.O., Johnson, B.B., Wells, J.D., Brooks, P., (2003), Characterization of carbonaceous combustion residues. I. Morphological, elemental and spectroscopic features, *Chemosphere* 51, p. 785
- Forbes, M. S., Raison, R. J., and Skjemstad, J. O., (2006), Formation, transformation and transport of black carbon (charcoal) in terrestrial and aquatic ecosystems, *Sci. Total Environ.*, 370, pp.190–206.
- Freudenberg, K., Chen, C.-L., Harkin, J.M., Nimz, H., Renner, H., 1965. Observations on lignin. *Chemical Communications*, (London) 1965, 224–225.
- Glaser, B., Haumaier, L., Guggenberger, G., and Zech, W., (1998), Black carbon in soils: the use of benzenecarboxylic acids as specific markers, *Org. Geochem. Vol. 29, No. 4*, pp. 811-819.
- Goldberg, E. D., (1985), Black carbon in the environment: properties and distribution. *Environmental science and technology* (New York), 0194-0287
- Gustafsson, Ö., Gschwend, P.M., (1997), Soot as a strong partition medium for polycyclic aromatic hydrocarbons. *Molecular Markers in Environmental Geochemistry*, Eganhouse, R.P., American Chemical Society: Washington, DC, USA, pp. 365-381.
- Hammes, K., Schmidt, M. W. I., Smernik, R. J., Currie, L. A., Ball, W. P., Nguyen, T. H., Louchouart, P., Houel, S., Gustafsson, Ö., Elmqvist, M., Cornelissen, G., Skjemstad, J. O., Masiello, C. O., Song, J., Peng, P., Mitra, S., Dunn, J. C., Hatcher, P. G., Hockaday, W. C., Smith, D. M., Hartkopf-

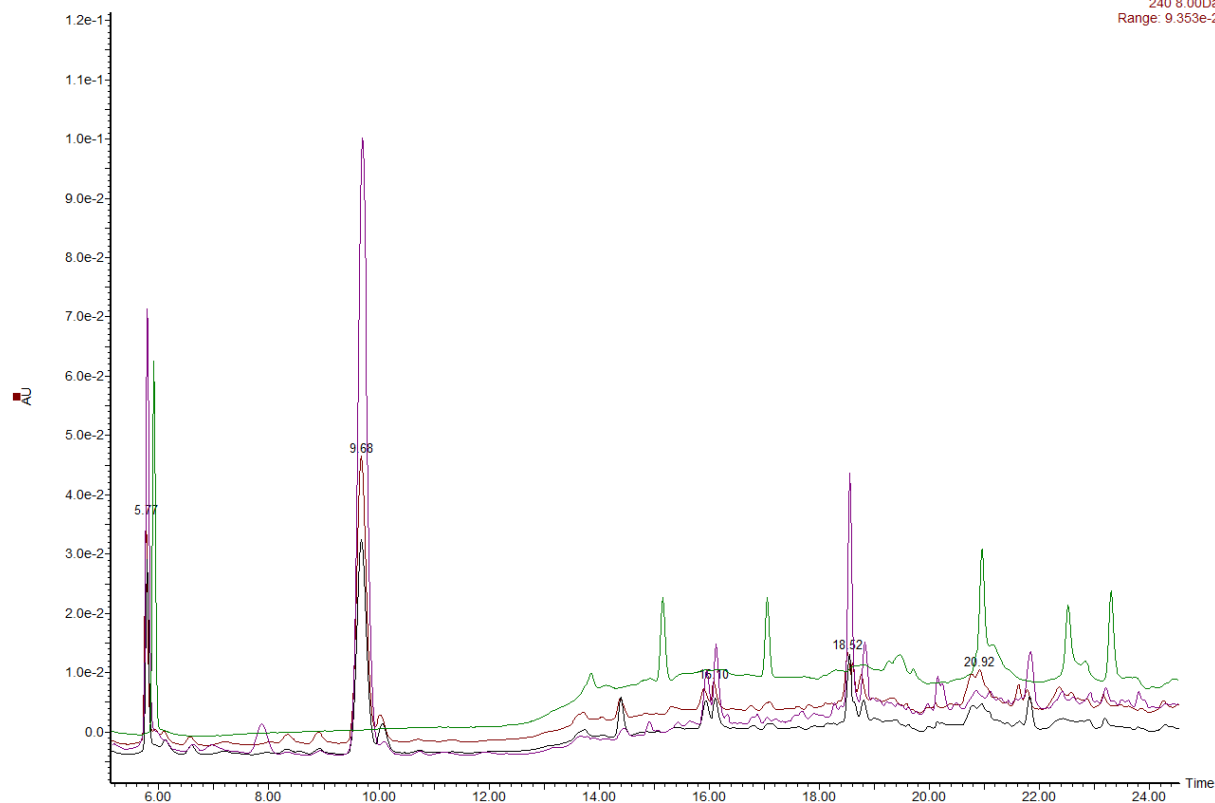
- Fröder, C., Böhmer, A., Lüer, B., Huebert, B. J., Amelung, W., Brodowski, S., Huang, L., Zhang, W., Gschwend, P. M., Flores-Cervantes, D. X., Largeau, C., Rouzaud, J.-N., Rumpel, C., Guggenberger, G., Kaiser, K., Rodionov, A., Gonzalez-Vila, F. J., Gonzalez-Perez, J. A., de la Rosa, J. M., Manning, D. A. C., López-Capél, E., Ding L., (2007), Comparison of quantification methods to measure fire-derived (black/elemental) carbon in soils and sediments using reference materials from soils, water, sediment and the atmosphere, *Global Biogeochemical Cycles*, vol 21, GB3016, doi: 10.29/2006GB002914 (Melanoidin at p. 4).
- Harris, D., C., (2003), *Quantitative Chemical Analysis*, 6:th edition, Removal of Melanoidin from Wastewater in Sugar Factories by Continuous Foam Fractionation Column. Student ID #: 97-1297-2 Name: SATO, Kosuke Supervisor: EGASHIRA, Ryuichi.
- Hedges, J. I., Eglinton, G., Hatcher, P.G., Kirchman, D.L., Arnosti, C., Derenne, S., Evershed, R.P., Kögel-Knabner, I., de Leeuw, J.W., Littke, R., Michaelis, W., Rullkötter, J., (2000). The molecularly-uncharacterized component of nonliving organic matter in natural environments. *Organic Geochemistry* 31, 945– 958.
- Herring, J., R., (1977). Charcoal fluxes into Cenozoic sediments of the North Pacific: unpublished Ph.D. thesis, University of California at San Diego, California, p. 105.
- Hansen, J. E., Sato, M., Ruedy, R., Lacis, A., and Oinas, V., (2000), Global warming in the twenty-first century: An alternative scenario, *P. Natl. Acad. Sci. USA*, 97 (18), 9875-9880.
- Jacobson, M. Z. (2000), A physically-based treatment of elemental carbon optics: Implications for global direct forcing of aerosols, *Geophys. Res. Lett.*, 27 (2), 217-220, doi: 10.1029/1999GL010968.
- Koehlmans A.A., Jonker, M.T.O., Cornelissen, G., Bucheli, T.D., Van Noort, P.C.M., Gustafsson, Ö., (2006), Black carbon: The reverse of its dark side, *Chemosphere* 63, p. 365
- Kuhlbusch T.A.J., Crutzen, P.J., (1996), Black carbon, the global carbon cycle, and atmospheric carbon dioxide. *Biomass Burning and Global Change*; Levine J.S., Ed.; MIT Press: Cambridge, MA, USA, 1996, pp. 161-169.
- Masiello C. A., Druffel, E. R. M., Currie, L. A., (2002), Radiocarbon measurements of black carbon in aerosols and ocean sediments. *Geochim. Cosmochim. Acta*, 66, pp. 1025-1036.
- Masiello C. A., (2004), New directions in black carbon organic geochemistry, Department of Earth Science, Rice University, Houston, United States, *Marine Chemistry* 92, pp. 201-213.
- Mattila T.J., Verta, M., (2008), Modeling the importance of biota and black carbon as vectors of polybrominated diphenyl ethers (PBDEs) in the baltic sea ecosystem. *Environ. Sci. Technol.*, 42, pp. 4831-4836.
- Meredith W., Ascough, P. L., Bird, M. I., Large, D. J., Snape, C. E., Sun, Y., Tilston, E. L. (2012), Assessment of hydrolysis as a method for the quantification of black carbon using standard reference materials. *Geochemica et Cosmochimica* 97, pp. 131-147.
- Nguyen, T. H., Brown, R. A., Ball, W. P., (2004), An evaluation of thermal resistance as a measure of black carbon content in diesel soot, wood char, and sediment. *Org. Geochem.* 35, pp. 217-234.
- Poot, A., Quik, J. T. K., Veld, H., Koelmans, A. A., (2009), Quantification methods of Black Carbon: Comparison of Rock-Eval analysis with traditional methods, *Journal of Chromatography A*, 1216, pp. 613-622
- Rosen, H., Hansen, A. D. A., Gundel, L., Novakov, T. (1978), Identification of the optically absorbing component in urban aerosols, Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720
- Roth, P. J., Lehndorff, E., Brodowski, S., Borneman, L., Sanchez-Garcia, L., Gustafsson, Ö., Amelung, W., (2012), Differentiation of charcoal, soot and diagenetic carbon in soil: Method comparison and perspectives, *Organic Geochemistry* 46, pp. 66-75, (p. 70 for BPCA numbers).
- Penner, J. E., Eddleman, H., Novakov, T., (1993), Towards the development of a global inventory of black carbon emissions. *Tmos. Environ.*, 27, pp. 1277-1295.
- Schmidt M. W. I., Skjemstad, J. O., Ghert E., Kögel-Knabner I., (1999), Charred organic carbon in German chernozemic soils, *European Journal of Soil Science*, 50, pp. 351-365.
- Schmidt M. W. I., (2000), Black carbon in soils and sediments: Analysis, distribution, implications, and current challenges. *Global Biogeochemical Cycles*, Vol. 14, No. 3, pp. 777-793.

- Schmidt M. W. I., Skjemstad, J. O., Szimczik, C. I., Glaser, B., Prentice, K. M., Gelinás, Y., Kuhlbusch, A. J., (2001), Comparative analysis of black carbon in soils, *Global Biogeochemical Cycles*, vol. 15, No 1, pp. 163-167.
- Schmidt M.W.I., Masiello, C.A., Skjemstad, J.O., (2003), Final recommendations for reference materials in black carbon analysis, *EOS* volume 84, Issue 52, p. 852.
- Schmidt M. W. I., (2005), *Black Carbon in Soils Looking for the Missing Carbon*, Universität Zürich, Zürich, Switzerland
- Schneider, M.P.W., Hilf M., Vogt, U.F., Schmidt, M.W.I., (2010), The benzene polycarboxylic acid (BPCA) pattern of wood pyrolyzed between 200 °C and 1000 °C, *Organic Geochemistry* 41, pp. 1082-1088
- Shackley, S., Sohi, S., Haszeldine, S., Manning, D., Masek, O., (2009), Biochar, reducing and removing CO<sub>2</sub> while improving soils: A significant and sustainable response to climate change? Evidence submitted to the Royal Geo-engineering Climate Enquiry in December 2008 and April 2009. UKBRC Working Paper 2
- Shresta, G., Traina S. J., Swanston, C. W., (2010), Black carbon's properties and role in the environment: A Comprehensive review. *Sustainability* 2010, 2, doi:10.3390/su2010294. pp. 294-320
- Simpson, M. J., Hatcher, P.G., (2004), Overestimates of black carbon in soils and sediments. *Naturwissenschaften*, 91, pp. 436-440.
- Skjemstad, J. O., Taylor, J. A., Smernik, R. J., (1999), Estimation of charcoal (char) in soils. *Commun. Soil Sci. Plant Anal.*, 30(15&16), pp. 2283-2298.
- Snyder, L. R., Kirkland, J. J., Glajch, J. L., 1997, *Practical hplc method development*, second edition. Chapter 8.5.2.3.
- Vollhardt, K. P. C., Schore, N. E., 2007, *Organic chemistry, Structure and function*, fifth edition, p. 667.
- Wiedemeier, D. B., Hilf, M. F., Smittenberg, R. H., Haberle, S. G., Schmidt, M. W. I. (2013), Improved assessment of pyrogenic carbon quantity and quality in environmental samples by high-performance liquid chromatography, *Journal of Chromatography A*, 1304, pp. 246-250.
- Zhang R., Khalizov, A.F., Pagels, J., Zhang, D., Xue, H., McMurphy, P.H., (2008), Variability in morphology, hygroscopicity, and optical properties of soot aerosols during atmospheric processing. *PNAS*, 105, pp. 10291-10296
- Ziolkowski, L.A., Druffel, E.R.M., (2009), The feasibility of isolation and detection of fullerenes and carbon nanotubes using benzene polycarboxylic acid method, *Marine Pollution Bulletin* 59, pp. 213-218

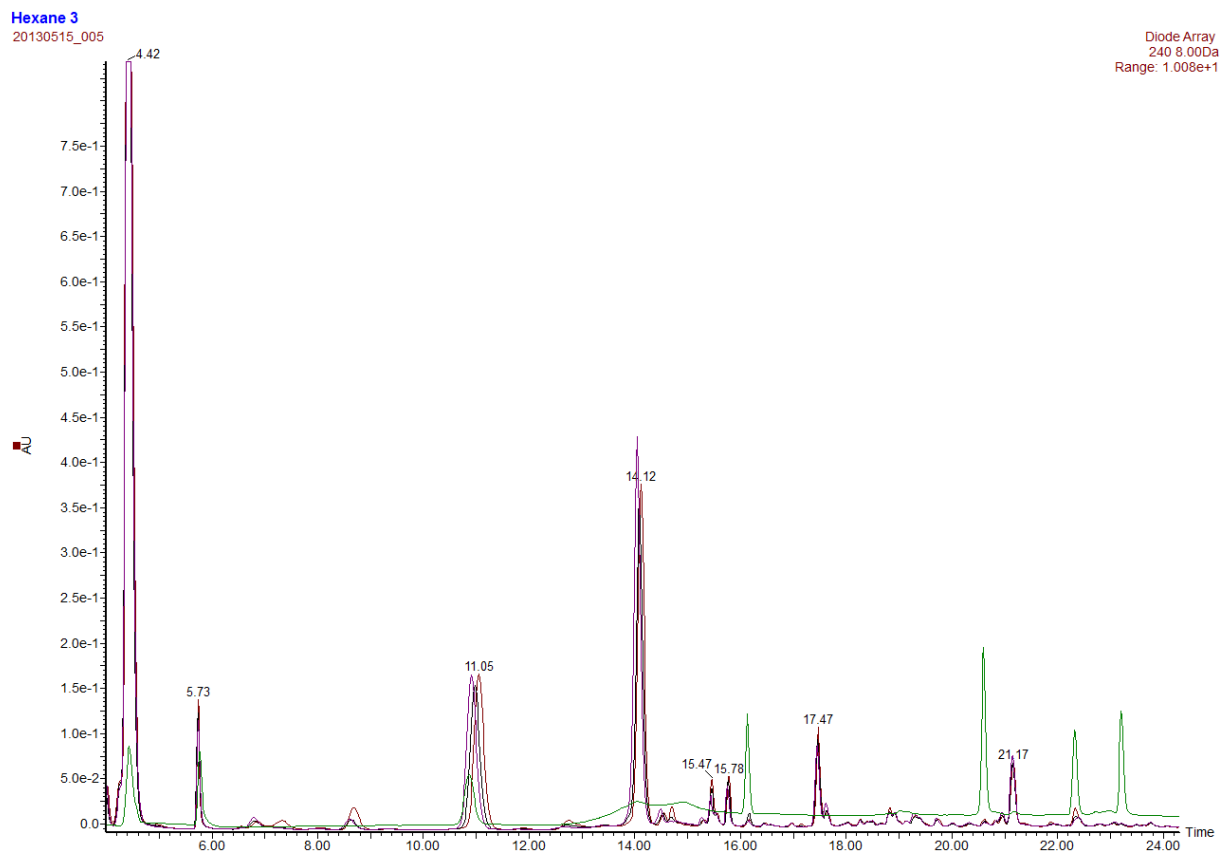
## 9. Appendices



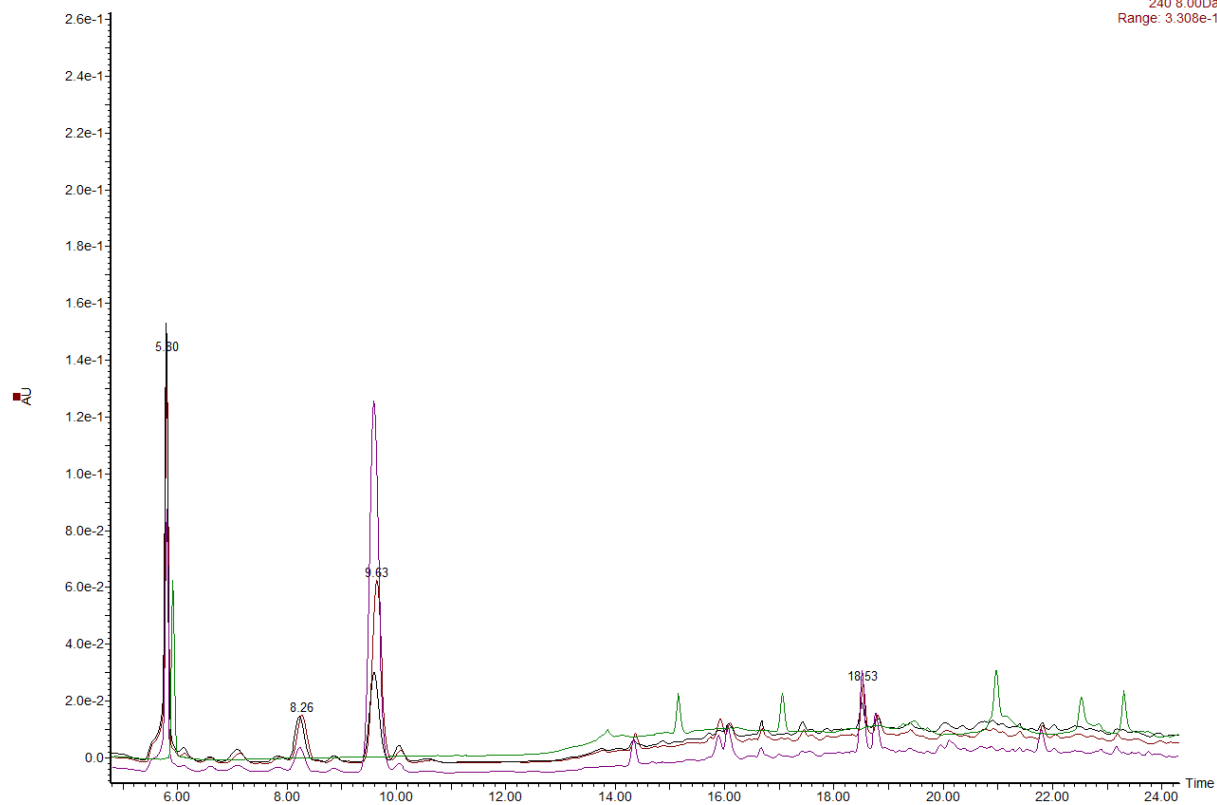
**Figure A1** Chromatograms of the oxidation products separated by HPLC for grass char, “Rice Straw” (black, purple, and red lines, 3 different replicates), compared to chromatogram of BPCA-standard mixture (green line).



**Figure A2** Chromatograms of the oxidation products separated by HPLC for wood char, “Chestnut Wood” (black, purple, and red lines, 3 different replicates), compared to chromatogram of BPCA-standard mixture (green line).



**Figure A3** Chromatograms of the oxidation products separated by HPLC for hexane, (black, purple, and red lines, 3 different replicates), compared to chromatogram of BPCA-standard mixture (green line).



**Figure A4** Chromatograms of the oxidation products separated by HPLC for melanoidin (black, purple, and red lines, 3 different replicates), compared to chromatogram of BPCA-standard mixture (green line).

**Table A1** The diagram shows the BPCA deviation from the BPCA calibration curve. "A5", "A6" abbreviations in the table are chernoziem soil samples by Wiedemeier, Zürich; "C" abbreviation is for chernoziem soil samples prepared in this study, with different injection volumes stated (all other injection volumes are 10 µL). "RS"- rice straw. "CW" – rice straw. "H" – chestnut wood. "M" – hexane. "M" – melanoidin. Yellow colored boxes (above) indicates that the concentration of BPCA was above the calibration limit (>16 ng). Green boxes (ok) indicates that the amount of detected BPCA is within the calibration range (2-16 ng). Blue colored box indicates that the amount of BPCA detected is below the calibration range (<2ng).

BPCA	A5	A6	C 5µL	C 5µL	C 10 µL	C 10 µL	C 10µL	C 15µL	C 20µL	C 20µL	C 20µL	C 25µL
B6CA	above	above	ok	ok	ok	above	above	above	above	above	above	above
B5CA	above	above	ok	ok	below	ok	ok	ok	ok	ok	ok	ok
B4CA-1,2,4,5	ok	ok	below	below	below	ok	ok	ok	ok	ok	ok	ok
B3CA-1,2,4	ok	ok	no data	below	below	below	ok	below	ok	no data	below	below
B3CA-1,2,3	ok	ok	below	ok	below	ok	ok	ok	ok	below	below	below
B3CA-1,3,5	below	ok	below	ok	below	ok	below	ok	ok	below	below	below
<b>BPCA</b>	<b>RS 1</b>	<b>RS 2</b>	<b>RS 3</b>	<b>RS 4</b>	<b>RS 5</b>	<b>CW 1</b>	<b>CW 2</b>	<b>CW 3</b>	<b>CW 4</b>	<b>CW 5</b>	<b>CW 6</b>	
B6CA	above	ok	ok	ok	ok	above	ok	ok	ok	ok	ok	ok
B5CA	ok	ok	below	below	ok	ok	no data	below	below	below	below	below
B4CA-1,2,4,5	below	below	below	ok	below	below	below	below	below	below	below	below
B3CA-1,2,4	below	below	below	ok	below	below	below	below	below	below	below	below
B3CA-1,2,3	below	ok	below	ok	ok	below	below	below	ok	below	below	below
B3CA-1,3,5	below	below	below	ok	ok	below	below	below	ok	ok	ok	ok
<b>BPCA</b>	<b>H 1</b>	<b>H 2</b>	<b>H 3</b>	<b>M 1</b>	<b>M 2</b>	<b>M 3</b>	<b>M 4</b>	<b>M 5</b>				
B6CA	above	above	above	above	above	above	ok	above	above	above	above	above
B5CA	above	above	above	no data	no data	no data	no data	no data	no data	no data	no data	no data
B4CA-1,2,4,5	above	above	above	no data	no data	no data	ok	no data	no data	no data	no data	no data
B3CA-1,2,4	above	above	above	no data	no data	no data	below	no data	no data	no data	no data	no data
B3CA-1,2,3	ok	ok	above	no data	no data	no data	no data	no data	no data	ok	ok	ok
B3CA-1,3,5	ok	above	ok	no data	no data	no data	no data	no data	no data	no data	no data	ok

**Table A2.** BPCA concentration, carbon content, and calculations of amount of pyrogenic carbon per total organic content in samples.

	Wiedemeier chernozem A5 Daniel		Wiedemeier chernozem A6 Daniel		Hexane1		Hexane2		Hexane 3	
	BPCA, ng	C-content, ng	BPCA, ng	C-content	BPCA, ng	C-content	BPCA, ng	C-content	BPCA, ng	C-content
B6CA	211,9888	89,2964	226,0322	95,21194	1516,258	638,6959	1640,889	691,1942	2264,589	953,9165
B5CA	129,3313	57,30964	137,9673	61,13643	275,6006	122,1249	286,7113	127,0483	418,8263	185,5914
B4CA-1,2,4,5	18,51735	8,751207	19,55678	9,242435	28,77632	13,59954	40,69369	19,23163	64,52659	30,49494
Missing B4CA's		17,50241		18,48487		27,19909		38,46326		60,98988
B3CA-1,2,4	5,418796	2,787492	5,682974	2,923389	1,281992	0,659472	2,248053	1,156425	5,229108	2,689915
B3CA-1,2,3	7,099212	3,651918	7,209148	3,708471	14,35793	7,385888	20,04343	10,31058	43,89095	22,57802
B3CA-1,3,5	1,623312	0,835051	3,01431	1,550597	1,962257	1,009408	1,658701	0,853255	2,465607	1,268337
Total, ng	373,9788	180,1341	399,4628	192,2581	1838,237	810,6742	1992,244	888,2576	2799,527	1257,529
BPCA total in vial (ug)		18,01341		19,22581		81,06742		88,82576		125,7529
sample weight (mg)		50		50		1		1		1
C in sample (mg)		0,965		0,965		0,9213		0,9213		0,9213
BPCA-C / sample ug / mg		18,66675		19,92312		87,99242		96,41351		136,4951
ng BPCA C per 1 mL		18013,41		19225,81		81067,42		88825,76		125752,9
g BPCA C per 1 g Sample		0,00036		0,000385		0,081067		0,088826		0,125753
BC per TOC		0,018667		0,019923		0,087992		0,096414		0,136495
%-BC of TOC		0,024369		0,02498		0,073		0,079		0,111
g BC per 1 kg Sample		0,360268		0,384516		81,06742		88,82576		125,7529
g BC per kg TOC		18,66675		19,92312		87,99242		96,41351		136,4951

**Table A3.** BPCA concentration, carbon content, and calculations of amount of pyrogenic carbon per total organic content in samples.

	Chernozem 1 (06/19) 5uL		Chernozem 2 (06/19) 10 uL		Chernozem 3 (06/19) 15 uL		Chernozem 4 (06/19) 20 uL		Chernozem 5 (06/19) 25 uL	
	BPCA, ng	C-content, ng	BPCA, ng	C-content, ng	BPCA, ng	C-content, ng	BPCA, ng	C-content, ng	BPCA, ng	C-content, ng
B6CA	11,45824	4,826575	12,19119	5,135314	28,80997	12,13567	97,00005	40,85949	79,54216	33,50568
B5CA	1,874339	0,830562	0,993925	0,440431	3,173919	1,406436	10,2479	4,541076	11,70217	5,185495
B4CA-1,2,4,5	0,247669	0,117047	0,753796	0,35624	2,375053	1,122438	4,384198	2,07195	6,298713	2,97674
Missing B4CA's	0,234094	0,712481								
B3CA-1,2,4	0,172177	0,08857	2,158981	1,110605	3,954395	2,034187	0,420194	0,216153	0,669182	0,344235
B3CA-1,2,3	0,040981	0,021081	0,046134	0,023732	1,661064	0,854471	0,089078	0,045823	0,759736	0,390817
B3CA-1,3,5	0,043515	0,022385	0,643068	0,330802	0,275262	0,141598	1,013967	0,521596	0,537979	0,276743
Total, ng	13,33258	6,140313	14,58198	8,109604	34,6342	19,93968	112,6461	52,39999	99,50994	48,63319
BPCA total in vial (ug)	1,228063		0,81096		1,329312		2,619999		1,945328	
sample weight (mg)	50		50		50		50		50	
C in sample (mg)	0,965		0,965		0,965		0,965		0,965	
BPCA-C / sample ug / mg	1,272604		0,840374		1,377525		2,715025		2,015883	
ng BPCA C per 1 mL	1228,063		810,9604		1329,312		2619,999		1945,328	
g BPCA C per 1 g Sample	2,46E-05		1,62E-05		2,66E-05		5,24E-05		3,89E-05	
BC per TOC	0,001273		0,00084		0,001378		0,002715		0,002016	
%-BC of TOC	0,002064		0,00104		0,006115		0,0044		0,00303	
g BC per 1 kg Sample	0,024561		0,016219		0,026586		0,0524		0,038907	
g BC per kg TOC	1,272604		0,840374		1,377525		2,715025		2,015883	

**Table A4.** BPCA concentration, carbon content, and calculations of amount of pyrogenic carbon per total organic content in samples.

	Chernozem 1 (07/06) 5uL	Chernozem 2 (07/06) 10 uL	Chernozem 3 (07/06) 20 uL	Chernozem 4 (07/06) 20 uL	Chernozem 5 (07/06) 25 uL
	BPCA, ng	BPCA, ng	BPCA, ng	BPCA, ng	BPCA, ng
	C-content, ng	C-content, ng	C-content, ng	C-content, ng	C-content, ng
B6CA	17,9466	38,28553	73,45656	97,88715	13,86852
B5CA	4,181758	7,677483	17,28662	15,54282	2,522425
B4CA-1,2,4,5	0,842242	1,575855	2,273865	3,383373	0,431119
Missing B4CA's	0,796079	1,489483	2,149234	3,19793	0,407489
B3CA-1,2,4	0,177281	0,091196	0,097697	1,644964	0,105829
B3CA-1,2,3	1,963633	1,010116	1,486393	10,34134	0
B3CA-1,3,5	1,33642	0,68747	1,105026	4,642381	0,779643
Total, ng	26,44793	52,76642	110,9058	133,442	17,70753
BPCA total in vial (ug)	2,479122	2,445248	2,551417	3,073572	0,321053
sample weight (mg)	50	50	50	50	50
C in sample (mg)	0,965	0,965	0,965	0,965	0,965
BPCA-C / sample ug / mg	2,569038	2,533936	2,643955	3,185048	0,332698
ng BPCA C per 1 mL g BPCA C per 1 g Sample	2479,122	2445,248	2551,417	3073,572	321,0533
BC per TOC	4,96E-05	4,89E-05	5,1E-05	6,15E-05	6,42E-06
%-BC of TOC	0,002569	0,002534	0,002644	0,003185	0,000333
g BC per 1 kg Sample	0,00448	0,0036	0,00415	0,00526	0,00061
g BC per kg TOC	0,049582	0,048905	0,051028	0,061471	0,006421
	2,569038	2,533936	2,643955	3,185048	0,332698

**Table A5.** BPCA concentration, carbon content, and calculations of amount of pyrogenic carbon per total organic content in samples.

	Rice Straw 1 (06/19)		Rice Straw 2 (06/19)		Rice Straw 3 (06/19)		Rice Straw 1 (06/07)		Rice Straw 2 (06/07)	
	BPCA, ng	C-content, ng	BPCA, ng	C-content, ng	BPCA, ng	C-content, ng	BPCA, ng	C-content, ng	BPCA, ng	C-content, ng
B6CA	29,91603	12,60158	14,86072	6,259806	8,110605	3,416444	7,233384	3,046931	12,06214	5,080954
B5CA	2,164133	0,958976	1,59758	0,707924	0,607355	0,269133	1,245055	0,551712	1,705576	0,755779
B4CA-1,2,4,5	1,479352	0,699134	1,118261	0,528484	0,254602	0,120324	2,165369	1,023343	1,375728	0,650162
Missing B4CA's	1,398269	1,056969	1,056969	1,056969	0,240647	0,240647	2,046685	2,046685	1,300324	1,300324
B3CA-1,2,4	1,123413	0,577897	1,189518	0,611902	1,111382	0,571708	1,730512	0,890196	1,044791	0,537453
B3CA-1,2,3	1,363648	0,701476	1,143879	0,588425	0,06233	0,032063	7,180683	3,693828	9,671419	4,975092
B3CA-1,3,5	1,565418	0,80527	0,791325	0,407067	0,653719	0,336281	2,481756	1,276644	2,191771	1,127473
Total, ng	37,612	17,7426	19,51176	10,16058	9,626281	4,9866	22,03676	12,52934	28,05142	14,42724
BPCA total in vial (ug)	1,77426	1,016058	1,016058	1,016058	0,49866	0,49866	1,252934	1,252934	1,442724	1,442724
sample weight (mg)	1	1	1	1	1	1	1	1	1	1
C in sample (mg)	0,5914	0,5914	0,5914	0,5914	0,5914	0,5914	0,5914	0,5914	0,5914	0,5914
BPCA-C / sample ug / mg	3,000102	1,718055	1,718055	1,718055	0,843186	0,843186	2,118589	2,118589	2,439506	2,439506
ng BPCA C per 1 mL	1774,26	1016,058	1016,058	1016,058	498,66	498,66	1252,934	1252,934	1442,724	1442,724
g BPCA C per 1 g Sample	0,001774	0,001016	0,001016	0,001016	0,000499	0,000499	0,001253	0,001253	0,001443	0,001443
BC per TOC	0,003	0,001718	0,001718	0,001718	0,000843	0,000843	0,002119	0,002119	0,00244	0,00244
%-BC of TOC	0,00447	0,00247	0,00247	0,00247	0,00103	0,00103	0,00184	0,00184	0,00277	0,00277
g BC per 1 kg Sample	1,77426	1,016058	1,016058	1,016058	0,49866	0,49866	1,252934	1,252934	1,442724	1,442724
g BC per kg TOC	3,000102	1,718055	1,718055	1,718055	0,843186	0,843186	2,118589	2,118589	2,439506	2,439506

**Table A6.** BPCA concentration, carbon content, and calculations of amount of pyrogenic carbon per total organic content in samples.

	Chestnut W. 1 (06/19) C-content, BPCA, ng ng		Chestnut W. 2 (06/19) C-content, BPCA, ng ng		Chestnut W. 3 (06/19) C-content, BPCA, ng ng		Chestnut Wood 1 (06/07) BPCA, ng ng		Chestnut Wood 2 (06/07) BPCA, ng ng		Chestnut Wood 3 (06/07) BPCA, ng ng	
B6CA	28,03657	11,80989	5,631983	2,37237	6,10636	2,572192	14,30607	6,026169	18,24043	7,683449	12,99716	5,474815
B5CA	1,666929	0,738654	0,363174	0,160931	0,481412	0,213325	1,297225	0,57483	1,256775	0,556905	1,200175	0,531825
B4CA-1,2,4,5	1,015761	0,480043	0,912699	0,431337	0,570159	0,269454	0,993462	0,469505	1,492004	0,705114	1,233128	0,58277
Missing B4CA's		0,960087		0,862674		0,538908		0,93901		1,410227		1,16554
B3CA-1,2,4	1,760162	0,905448	2,253886	1,159425	4,678879	2,40687	0,025263	0,012996	0,19162	0,098572	0,227225	0,116887
B3CA-1,2,3	0,024048	0,012371	0,492012	0,253097	0,745012	0,383243	6,244264	3,212123	3,008515	1,547616	6,523766	3,355902
B3CA-1,3,5	1,054853	0,542629	0,428157	0,220249	0,441385	0,227053	3,65475	1,880046	2,934255	1,509415	4,446882	2,287528
Total, ng	31,77411	15,44912	6,06014	5,460082	6,547745	6,611047	26,52103	13,11468	27,1236	13,5113	26,62834	13,51527
BPCA total in vial (ug)	1,544912		0,546008			0,661105		1,311468		1,35113		1,351527
sample weight (mg)	1	1	1	1	1	1	1	1	1	1	1	1
C in sample (mg)	0,7444	0,7444	0,7444	0,7444	0,7444	0,7444	0,7444	0,7444	0,7444	0,7444	0,7444	0,7444
BPCA-C / sample ug / mg	2,075379		0,733488			0,888104		1,761778		1,815059		1,815592
ng BPCA C per 1 mL	1544,912		546,0082			661,1047		1311,468		1351,13		1351,527
g BPCA C per 1 g Sample	0,001545		0,000546			0,000661		0,001311		0,001351		0,001352
BC per TOC	0,002075		0,000733			0,000888		0,001762		0,001815		0,001816
%-BC of TOC	0,00351		0,00049			0,00054		0,00266		0,00284		0,00292
g BC per 1 kg Sample	1,544912		0,546008			0,661105		1,311468		1,35113		1,351527
g BC per kg TOC	2,075379		0,733488			0,888104		1,761778		1,815059		1,815592

**Table A7.** BPCA concentration, carbon content, and calculations of amount of pyrogenic carbon per total organic content in samples.

	Melanoidin 1 (06/19) C-content, ng		Melanoidin 2 (06/19) C-content, ng		Melanoidin 3 (06/19) C-content, ng		Melanoidin 1 (06/07) C-content, ng		Melanoidin 3 (06/07) C-content, ng	
	BPCA, ng	C-content, ng	BPCA, ng	C-content, ng	BPCA, ng	C-content, ng	BPCA, ng	C-content, ng	BPCA, ng	C-content, ng
B6CA	44,3285	18,67257	36,85764	15,5256	37,3111	15,71662	16,29939	6,865819	71,36758	30,06229
B5CA	0	0	0	0	0	0	0	0	0	0
B4CA-1,2,4,5	0	0	0	0	0	0	11,22156	5,303253	1,964305	0,928321
Missing B4CA's	0	0	0	0	0	0	10,60651	0	0	1,856641
B3CA-1,2,4	0	0	0	0	0	0	0	0	0	0
B3CA-1,2,3	0	0	0	0	0	0	0	0	3,22397	1,658448
B3CA-1,3,5	0	0	0	0	0	0	0,438292	0,225463	2,159474	1,110859
Total, ng	44,3285	18,67257	36,85764	15,5256	37,3111	15,71662	27,95924	23,00104	78,71533	35,61655
BPCA total in vial (ug)	1,867257		1,55256		1,571662		2,300104		3,561655	
sample weight (mg)	1		1		1		1		1	
C in sample (mg)	0,5413		0,5413		0,5413		0,5413		0,5413	
BPCA-C / sample ug / mg	3,449579		2,868207		2,903494		4,249222		6,579818	
ng BPCA C per 1 mL	1867,257		1552,56		1571,662		2300,104		3561,655	
g BPCA C per 1 g Sample	0,001867		0,001553		0,001572		0,0023		0,003562	
BC per TOC	0,00345		0,002868		0,002903		0,004249		0,00658	
%-BC of TOC	0,01789		0,00852		0,034332		0,00709		0,00903	
g BC per 1 kg Sample	1,867257		1,55256		1,571662		2,300104		3,561655	
g BC per kg TOC	3,449579		2,868207		2,903494		4,249222		6,579818	

## Part 2

### Preparation and maintenance of the HPLC instrument, Waters 2695

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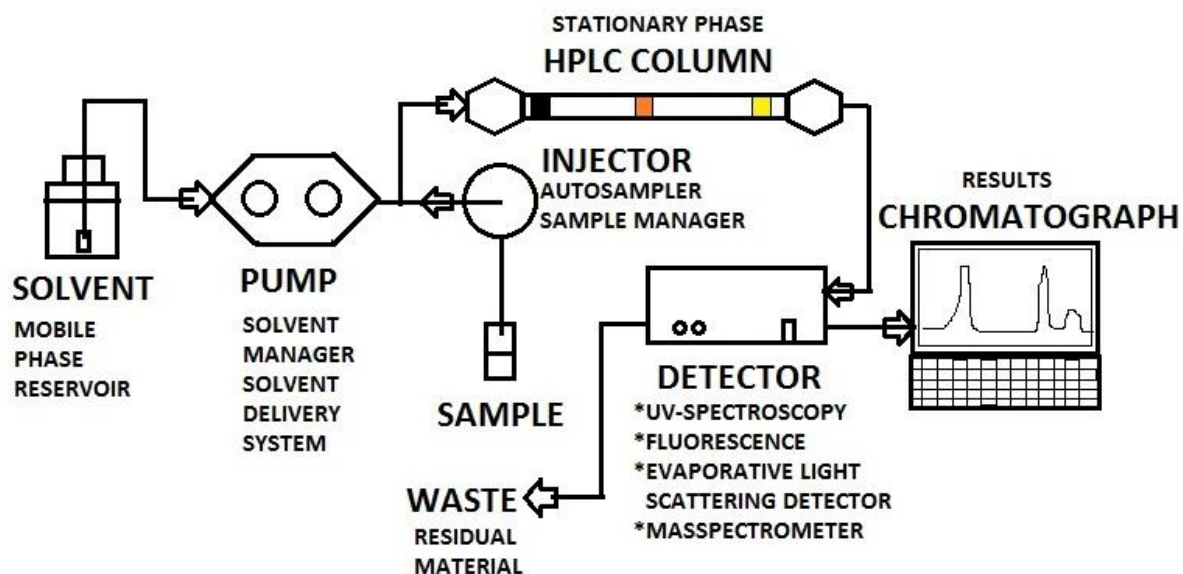
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# 1. Introduction

In this part of the study focus lies on the theory of high performance liquid chromatography, maintenance and preparation of the instrumentation, in this case, a separation module Waters 2695, equipped with diode array detector (DAD) Waters 996. Waters 2695 was chosen because there were two similar units (Waters 2695 and 2690) available at the moment at Stockholm University, while all other operational units were occupied by other workers. It was decided to extend this study, and repair one of the older units, to gain a deeper knowledge of how to operate and maintain an HPLC-system, and to be able to conduct pyrogenic carbon analysis.

## 1.1. Basic principles of high performance liquid chromatography

Liquid chromatography has been developed to be applied for compounds that are not suitable for gas chromatography (GC) due to the inadequate volatility properties of the material. The basic principle of *high performance liquid chromatography*, HPLC, is to separate different compounds depending on their polarity. Main components of an HPLC-system are a *solvent management system*, with a solvent reservoirs connected to it; a *sample injection valve*; an *autosampler*; a high-pressure chromatography *column*; a *detector*; and a computer equipped with a software to control operations, process and present results (Figure 1). A dissolved sample is mixed with solvents, or the mobile phase, in the solvent management system, and mixture is then pumped to a *HPLC-column* where separation occurs. Different polarity properties of the compounds makes it possible for them to *elute*, or in other words to be extracted from one another through the column. A column is packed with specific material depending on the nature of *analyte* – the compound needed to be analysed. Normally a HPLC-column is placed in an oven to sustain constant temperature. A sample solution is injected into the mixture of mobile phases before entering the column through an injection valve mechanism. An injection valve has interchangeable sample loops. Sample volume that can be injected is usually between 2 and 100  $\mu\text{L}$  (Harris, 2010). Stationary phase in a column appeals different compounds with different strength. Fastest compounds are more attached to the mobile phase, or the mixture of solvents, and will be the first one to leave a column and therefore have the fastest *retention time*,  $t_r$ . Slowest compounds are more attracted to the particles of the packing material in a column and will retain longer. A detector is used to analyse different compounds that elutes at specific retention times. Many different types of detectors are in use, most common for HPLC are spectroscopy instruments that measures the absorption at specific wavelength, which reflects the concentration of analyte in solution (Rouessac, 2007; Crawford Scientific, 2013). A spectrophotometer should be able to operate in the range of 185 to 900 nm (Rouessac, 2007). For this study Waters 996, an array diode detector has been used. A diode array makes it possible to measure several wavelengths simultaneously. The elute signal is recorded as peaks on the graph with higher intensity against the background noise, as the absorbance intensity (response) over time. The residual material is transported out of the detector to a waste container. The peak area is used to quantify the amount of compound with the help of reference standards.



**Figure 1.** Schematic picture of a HPLC system equipped with a detector and connected to a computer with a software to calculate and view the results.

Two main types of chromatography are in use for HPLC. *Normal-Phase Chromatography*, contains a polar stationary phase and less polar solvent. Eluent strength is bigger for a more polar phase. Less polar compounds elute before more polar compounds. Normal-phase chromatography operates less effectively if water is present in the mobile phase. In this study *Reversed-Phase Chromatography* was used for applied, which has a nonpolar stationary phase and more polar solvent. Compounds that are more polar will elute before less polar ones. High separation efficiency has made reverse phase (non-polar stationary phase with polar mobile phase) chromatography most widespread approach used within high performance liquid chromatography (Rouessac, 2007; Harris, 2010).

### 1.2. Column and stationary phase

A typical HPLC-column contains 40 % particles and 60 % solvent, or mobile phase. Column cover is usually made of stainless steel, highly resistant plastic materials or glass. Column volume is proportional to the square of column diameter. In most cases the stationary phase in the column is made of microporous (size range: 1,7-5  $\mu\text{m}$ ) particles of silica or stearyl alcohol octadecyl,  $C_{18}$ , with very high surface area. Pressure build up is often a result of clogging at the column inlet or outlet. Columns are sensitive to larger particles and gets damaged by clogging at the inlet. To protect the columns from degrading, solvent must be sonicated to remove bubbles. Solvent should also be filtered with a 0,5  $\mu\text{m}$  micro-filter, and centrifuged prior the injection (Rouessac, 2007; Harris, 2010).

### 1.3. Pump system, pressure and flow rate

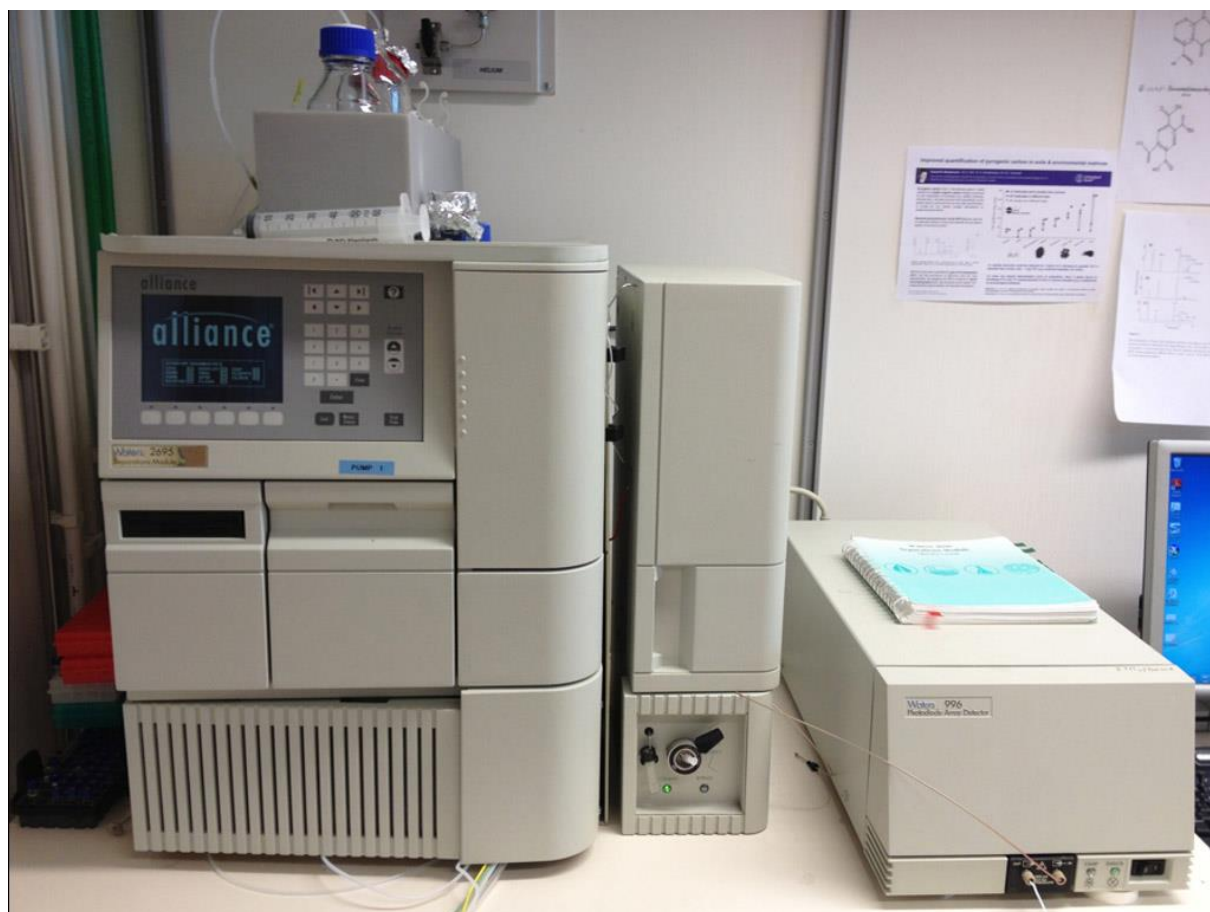
A HPLC system pump of a good quality must be able to sustain a stable flow without fluctuation at flow rates between 0,5-1 mL/min, and at pressure between 7-40 MPa or 70-400 bar (Harris, 2010; Snyder, 1997). Uneven flow cause problems for detection and may seriously affect the results if a sample contains relatively low concentrations of analyte. A typical HPLC delivering system pump has two pistons that works simultaneously and are

programmable to deliver a constant flow rate. A standard HPLC equipment can provide a solvent gradients from up to 4 different solvents. Temperature of the solvent must be equal to the column temperature; solvent must be then preheated before entering the column (Harris, 2010). Temperature differences results in distortion of peaks and varied retention times.

## 2. Maintenance and Preparation of Waters 2695 separation module

### 2.1. Prior the installation of Waters 2695

At the start of this project the first idea was to repair an older version of Waters 2695, model 2690. The instrument was not functioning. The fuses were checked on the instrument firstly, they were functional. The next step was to diagnose the power supply box. Conductivity was checked with a voltmeter and it was found that the power box was malfunctioning. It was decided to repair and use another model of instrument, a newer Waters 2695, which is a modified version of 2690. The instrument was able to start, electrical system was intact. For the next step, solvent supply tubing was installed from solvent bottles to the vacuum degasser. The tubes were marked for each different solvent. Waste line tubing from the degasser was installed and connected to a waste reservoir. The detector drip tray was also connected to the waste reservoir.



**Picture 1.** Waters 2695 Separation Module equipped with column heater, and Waters 996 Photodiode Array Detector (PAD)

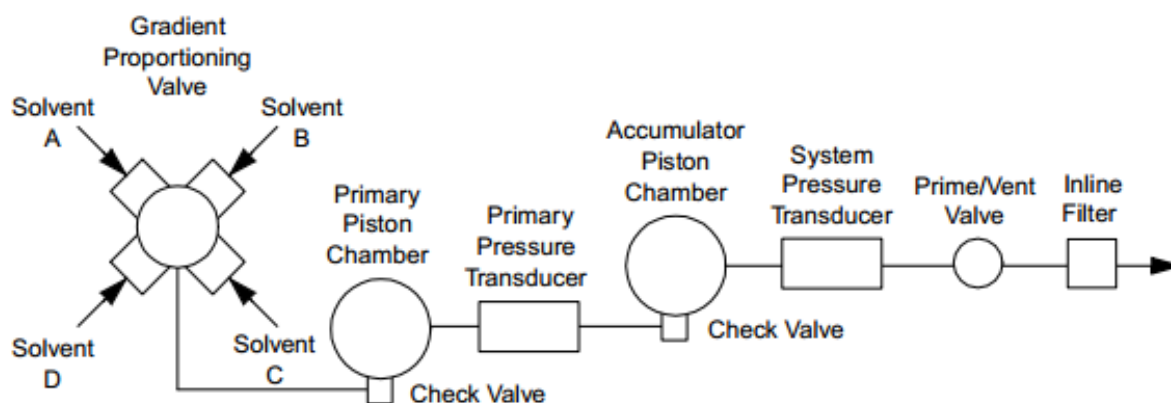
#### 2.1.1. Connection

To resolve the communication problems with the solvent management system, that were related to a damaged hardware, a newer GPIB Interface board was installed on the computer.

Proper drivers for the AT-GPIB/TNT board were installed; drivers must match the connection and the type of the board. Masslynx 4,1 was installed to connect and operate the instrument. In the Masslynx 4,1 software all the components such as separations module, column heater and detector could be recognized and configured properly.

### 2.1.3. Safety precautions

Leaking solvent inside the system was collected at the bottom tray and redirected to a waste bottle through an outlet. Solvent bottles were stored on a plastic drip tray to protect the instrument. A ventilation fan has been installed above the solvent bottles to collect any possible fumes that are emitted from acetonitrile.



**Figure 3.** Flow path through the solvent management system of Waters 2695. Source: Waters 2695 instructions manual, p. 39.

## 2.2 Detecting errors

After the proper fluidic connections were installed, and after the examination of the solvent management system, it was time to check the operational stability of the Waters 2695. The flow path through the solvent management system were examined, Figure 2. If the pressure is abnormally low in the system then there is probably a leak in the system according to Waters Corporation (2002).

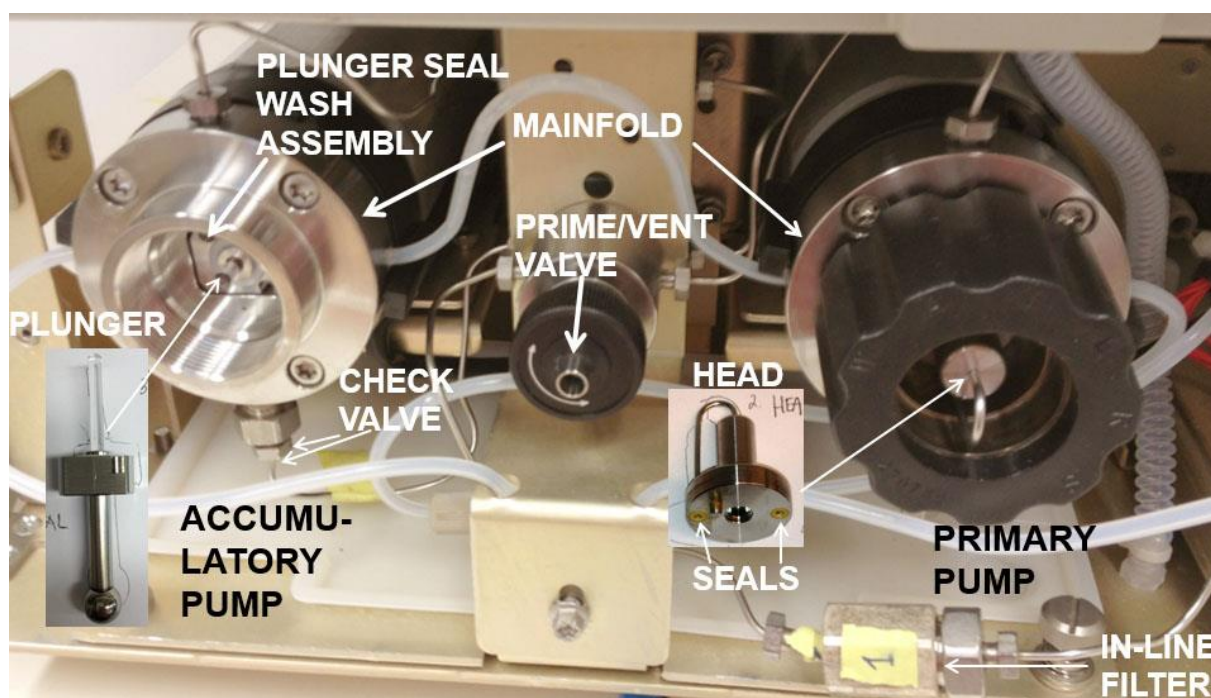
### 2.2.1 Flow stability and pressure

Back pressure was created by installing a test column. Firstly a dry prime was performed to get the solvent in to the system. Next goal was to check if the pressure is stable during a regular flow of the solvent. Pressure should not fluctuate more than 10 bars (145 psi) during a standard flow according to the manual of the instrument. In the beginning pressure fluctuated, which means there are bubbles in the system, or leakage. The first thing to deal with if the pressure fluctuates is to remove all bubbles in the system by purging/priming. Wet prime was performed at flow rate of 7,5 ml/min for 6 min (Waters Corporation, 2002). However the problem with the bubbles remained, and the instrument was left for wet priming overnight. To check if the flow rate was correct, the flow from the outlet tube was measured with a 5-ml volumetric flask by taking time sequences and manual notation of volume per time, it was noted that the flow was pulsating. Thorough examination of piston chamber revealed that the plunger was stuck in the plunger cone, causing the irregular solvent flow. It was decided to replace the whole pump-system with both primary and accumulator piston chambers with the

spare parts from Waters 2690. After the replacement the system was left over night for priming at flow rate 0,3 ml/min with sonicated ultrapure water. Pressure stabilized afterwards, and solvent management system worked properly.

### 2.2.2. Leaking

Many times leaking within the system management system was detected. Leaking usually occurred beneath the check valves, inside head or plunger, and in the tubing connections, Picture 2. To begin with, all the connections in the solvent manager system were checked, tubing parts, valves etc. were tightened and checked if they were attached correctly, specially inlet or outlet connections such as check valves, and in-line filter. Next step was to dismantle different parts of the pump system (plunger, head, seals, inlet check valves). All parts were examined, cleaned and sonicated in a mixture of isopropanol and water to remove the oxidation and precipitation rests. Seals that were damaged were replaced with new ones. After a successful service and installation of piston chamber parts, a dry prime of the solvent management system were performed to transport the solvent into the plunger space, and later wet prime to remove the bubbles.



**Picture 2.** Solvent Management System at Waters 2695. The accumulator piston chamber has been partly dismantled. Separate pictures of plunger and head to illustrate the inner components of the piston chambers.

### 2.2.3 Clogging and pressure built up

During this project the separation module has been clogged during an analysis once, which resulted in abnormal pressure built up and automatic shutdown of the solvent flow. To locate clogging different parts of the system has been disconnected systematically following the flow path to isolate the location of clogging. Column has been disconnected and only solvent management system was tested to see if the pressure was normal during the flow of solvent. Pressure levels were normal, but when the sample management system was reconnected,

pressure started to rise. Error was detected in outlet tubing leading to the column, the tubing was replaced, and the column inlet was cleaned, afterwards the problems with pressure build up were resolved.

### 2.3. Equilibrating the system

To prepare the system for each new analysis, it was flushed with solvent composition according to BPCA-method. The column was conditioned by running the solvent gradient without sample injection prior the analysis.

## 3. References

Harris D. C., (2010) Quantitative Chemical Analysis, Eight Edition. Daniel C. Harris. Michelson laboratory. China lake, California. W. H. Freeman and company, New York.

Rouessac F. & Rouessac A., (2007) Chemical Analysis, Modern Instrumentation Methods and Techniques, Second Edition, University of Le mans, France, (pp. 63-89, HPLC; pp. 167-203, spectroscopy)

Waters Corporation (2002), Waters 2695 Separation Module Operator's guide, Waters, 34 Maple Street, Milford, MA 01757, 71500269502, Revision B.

Waters Corporation (1997), Waters 996 PDA Detector Operator's Guide, Waters, 34 Maple Street, Milford, MA 01757, 0532021TP, Revision 0.

Snyder R. L., Kirkland J. J., Glajch J. L., (1997), Practical HPLC Method Development, Second edition, Section 1.4.2